A STUDY OF GENETIC VARIATION IN NATURAL POPULATIONS OF *DROSOPHILA MELANOGASTER*

by

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A thesis submitted for the degree
of Doctor of Philosophy of the
Australian National University

November, 1989

Declaration

The research work described in this thesis, ~~expect~~ except where acknowledged, is the original work of the author.

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November, 1989

Material presented in Chapter 2 and Chapter 4 has been published in *Heredity*:

Jiang, C., Gibson, J.B., Chen, H. 1989. Genetic differentiation in populations of *Drosophila melanogaster* from the People's Republic of China: comparison with patterns on other continents. *Heredity*, 62: 193-198.

Jiang, C., Gibson, J.B., Wilks, A.V. and Freeth, A.L. 1988. Restriction endonuclease variation in the region of the alcohol dehydrogenase gene: a comparison of null and normal alleles from natural populations of *Drosophila melanogaster*. *Heredity*, 60: 101-107.

Acknowledgements

I thank my supervisor Dr. John Gibson for his guidance, encouragement and patience over my past four year Ph.D course, and I thank my advisors Dr. George Miklos and Dr. Allan Freeth for sharing their knowledge so freely.

The scientists and students who work or had worked in our group are so friendly and helpful: Ann Wilks, Anh Cao, Darryl Reed and Dr. Peter Cooke provided excellent technical instruction and assistance; Jane Symonds, Dr. John Oakeshott, Birt Kohlmann, Peter Mathews, Dr. Adam Marchant and Dr. Dave Shaw provided valuable discussion.

I thank Ross Cunningham, Birt Kohlmann, David Sandiland, Steven Ball and Cathy Condon for their assistance in statistics and computer operation, Gary Brow and James Whitehead for the illustrations for my thesis.

I thank my Chinese colleague Huazhong Chen and Professor C.C.Tan for their assistance in collecting flies from China.

I thank Dr. Adam Marchant, Jane Symonds and Jeremy Brunhl for the help in proofreading the manuscript.

I am grateful for the financial support: for one year from the state educational committee of Poeple's Republic of China and for three years from the Australian National University Postgraduate Scholarship.

Finally I thank my wife Ye Chen for her encouragement and pleasant company in Canberra.

Abstract

This thesis is concerned with genetic geographic differentiation in natural populations of *Drosophila melanogaster*.

An electrophoretic survey has been carried out of allozyme frequencies at eight loci in eight natural populations from China. The Chinese data were collated with the data from Japanese populations. The analyses showed that the frequencies of $G6pd^F$, $Est\text{-}6^{1.00}$ and Adh^S were significantly correlated with latitude. Whilst the variation in $Est\text{-}6^{1.00}$ was opposite to that previous reported, these results showed the generality of latitudinal clines of Adh^S and $G6pd^F$ frequencies in the Chinese populations of *Drosophila melanogaster*. Previous work had showed that at least Adh^S, $G6pd^F$, $Est\text{-}6^{1.00}$, Odh^F and Pgd^F frequencies varied latitudinally in North America, Eurasia and Australasia.

104 and 90 second isochromosome lines were extracted from seven Australian and six Chinese populations respectively, along the *Adh* cline. Restriction mapping of these chromosomes indicated that there is no consistent relationship between restriction endonuclease variation and latitude, except for a 0.2kb deletion which decreases in frequency with distance from the equator in the Chinese populations.

Overall, the levels of nucleotide substitution and haplotype diversity are higher in Australian populations than in Chinese populations. The analyses of restriction endonuclease variation frequencies showed that the populations in the island of Tasmania differ from those on the Australian mainland. The level of heterozygosity is higher for the haplotypes with Adh^S than for those with Adh^F. Chromosomes bearing Adh^F heat resistant alleles have the lowest heterozygosity, reflecting the history of alleles suggesting the recent origin of these alleles.

Ten different insertions, detected in the Australian and Chinese populations, were cloned and screened with known mobile elements. Two insertions, I(0.28) and I(0.4) were found to be homologous in sequence

to mobile elements *B104B* and *F101* respectively. None of the insertions occurred in the *Adh* coding region.

Amongst chromosomes assayed for ADH activity most had normal intact *Adh* gene, only one null and one partial duplicated *Adh* gene were found in 104 Australian and 90 Chinese lines. ADH activity assays on the Australian and Chinese population samples indicated that the levels of ADH activity vary between different populations. The ADH activity levels in populations depend on the frequencies of Adh^F since on average the ADH activity of Adh^F alleles is twice as high as in Adh^S alleles. The mean ADH activities of the Adh^F and Adh^S bearing chromosomes vary between different geographic regions. Estimates of amount of ADH protein in 52 high and low ADH activity lines of Adh^F and Adh^S showed that both the catalytic efficiency and the amount of ADH protein make contributions to the difference in mean activity between Adh^F and Adh^S.

There is no consistent relationship between restriction endonuclease variants and ADH activity, except that some large insertions in the functional region of the *Adh* gene affect the expression of the gene. A significant linkage disequilibrium between the restriction site variant *Bam*HI(-7.2) and the Adh^S allele was found in most populations studied, and 5′ recombination involving this site may affect ADH activity in some cases.

A comparison of the restriction endonuclease maps of normal and null *Adh* alleles showed that twelve *Adh* null alleles from three Tasmanian populations share the same haplotype as four normal Adh^S alleles. This suggested that the Tasmanian *Adh* null alleles have a common origin.

A relatively high frequency of Adh^F heat resistant alleles was found in the southern Chinese populations, and these alleles share the characteristics of heat resistant alleles previously found in

Australia, America and Europe. It is possible that the Adh^F heat resistant allele originated in southern China, and then spread world-wide.

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CHAPTER 1

GENERAL INTRODUCTION

Chapter 1.General Introduction

My work in this thesis is concerned with genetic variation in natural populations of *Drosophila melanogaster.*

The application of the technique of protein gel electrophoresis first revealed large amounts of genetic variation in natural populations (Harris, 1966; Lewontin and Hubby, 1966). This technique made it possible to investigate directly levels of heterozygosity in natural populations. Over the next ten years investigations of allozyme variation in natural populations of animals and plants studied 243 species (Nevo, 1978). To date more than 1000 unrelated species worldwide have been studied (Nevo, 1989).

The large amounts of genetic variation found in natural populations (about 30% of loci were found to be polymorphic, Lewontin 1985) have been explained on the basis of two controversial theories: the neutralist and selectionist viewpoints. The neutralists claimed that the variation observed is essentially composed of non-selective mutations whose frequencies in natural populations are a consequence of random genetic drift and migration. They argued that since nearly all new mutations are deleterious, these mutations are eliminated by natural selection from natural populations. The variation that remains polymorphic consists of transient alleles of no adverse fitness effects, or slightly deleterious, effect on the organism. The selectionists argued that the observed variation is held in stable balance by forces of natural selection. The study of geographic patterns of genetic variation in natural populations became an important topic in attempts to explain the variation.

Analyses of the available data (Nevo 1978; 1984; 1989) have suggested that the amounts of genetic polymorphism and heterozygosity

vary non-randomly between loci, populations, species, habitats and life zones, and are correlated with ecological heterogeneity. Natural selection may often be the major determinant of genetic population structure and differentiation. *Drosophila* species show significantly higher levels of polymorphism and heterozygosity than most other species studied (Kojima *et al.*, 1970; Richmond, 1972; Ayala and Tracey 1974; Ayala *et al.*, 1974, 1974 a, b). The mean polymorphism (P) for *Drosophila* species is 0.431, while for other species it is 0.263; the mean heterozygosity (H) for *Drosophila* is 0.14, for other species it is 0.074 (Nevo 1978).

Drosophila melanogaster occurs worldwide associated with human habitation. It is found predominantly in domestic habitats, such as gardens, orchards, vineyards and garbage dumps. This species has been extensively studied and the polytene chromosomes and linkage maps are well known (Ashburner and Novitski, 1976; Lemeunier *et al.*, 1986) and genetic manipulation is possible. These advantages make *Drosophila melanogaster* good material for the study of genetic variation in natural populations.

According to previous work (Lemeunier *et al.*, 1986, David and Capy, 1988) a good deal of the genetic variation in *Drosophila melanogaster* populations shows geographic differentiation or latitudinal clines (table 1.1). This variation includes morphological, physiological, and behavioral traits, and particularly allozyme frequencies, chromosomal inversions etc. The variation in allozyme frequencies and chromosomal inversions directly reflect the genetic structure of the populations, whilst the genetic components of other variables are more complex, although they might often have some direct relationship to natural selection and adaptation.

Explanations for any of the geographic differentiation found in *Drosophila melanogaster* could involve the effects of migration, of geographic isolation or of some environmental selective factors (Endler 1977).

The species *Drosophila melanogaster* is believed to have arisen in central Africa 2-3 million years ago (David and Capy, 1988; Lachaise *et al.*, 1988). The evidence for this is that: (1)six out of eight members of *Drosophila melanogaster* species group are endemic to the Afrotropical region, (2)most of the polymorphic alleles found from the populations from other parts of the world have been found in populations of the Afrotropical region, and (3)the populations on the Afrotropical mainland are apparently genetically sub-divided, which is expected in long established populations between which there is restricted gene flow (David and Capy, 1988). The colonization of Eurasia by *Drosophila melanogaster* from central Africa without the agency of man occurred at least 10-15 thousand years ago (Lachaise *et al.*, 1988). The colonization of America and Australia by *Drosophila melanogaster* from Afrotropical and Eurasia probably occurred via the agency of modern man only a few centuries ago (Capy *et al.*, 1986; Jallon and David, 1988).

Thus some of the observed geographic genetic differentiation in *Drosophila melanogaster* may be explained as a result of historical migration. Suppose, for example, that the flies which migrate from one population to a new habitat have a lower frequency of a certain variant than in the original population. The frequency of the variant would be lower in the newly established population. If this procedure of migration is repeated, there would be a cline of the variant between the original population and the newly established populations. The distribution patterns of *P* element and sigma virus in *Drosophila*

melanogaster seem to be examples of this process - the frequencies of *P* elements and virus type II decrease with increasing distance from the ancestral locations (North America for *P* element, Anxolabehere *et al.*, 1985; David and Capy, 1988; France for virus type II, Fleuriet, 1986).

A second explanation for the geographic genetic differentiation in *Drosophila melanogaster* is geographic isolation. A good example of this is the distribution of mitochondrial DNA haplotypes in *Drosophila melanogaster*. Variation of mtDNA in *Drosophila melanogaster* in different geographic populations was surveyed by Hale and Singh (1987). They used eleven restriction endonucleases to survey the RFLPs (restriction fragment length polymorphisms) of mtDNA extracted from 92 isofemale lines from 18 populations from North America, Europe, Africa, South America, Australia and Asia. Four of these restriction endonucleases had polymorphic sites. In total 24 haplotypes were observed, of which 18 were unique to single populations and reached high frequencies in many populations without being observed in neighbouring populations. Mutationally close variants showed geographic clumping, suggesting local differentiation of mtDNA in populations of *Drosophila melanogaster*. If the populations were considered as New-World (Eastern Hemisphere) and Old-World (Western Hemisphere), then the Old-World and New-World are differentiated, with the predominant Old-World haplotype being virtually absent from the New-World. There is further population differentiation at the continent level, as the samples from France and England shared no haplotypes. The mtDNA data indicate a species history of colonization of new localities via bottlenecks and subsequent divergence of new local haplotypes (Hale and Singh, 1987).

A third explanation for the geographic differentiation of genetic variation is that the genetic variation is maintained by natural selection. Latitudinal clines of chromosomal inversions and allozyme frequencies have been claimed to arise from the results of environmental selection (Oakeshott *et al.*, 1981, Knibb, 1982; Oakeshott *et al.*,1982; Singh *et al.*, 1982; Oakeshott *et al.*, 1983a,b; Singh and Rhomberg, 1987). *Drosophila melanogaster* is highly polymorphic for chromosome inversions. The first survey of natural populations of *Drosophila melanogaster* for inversions by cytological methods was carried out by Dubinin (1937). 362 different inversions have been described (Lemeunier *et al.*,1986), and the majority of the inversions (316) are paracentric. Four inversions were found to be cosmopolitan: *In(2L)t, In(2R)NS, In(3L)P and In(3R)P* - they occur in most natural populations, often at a frequency greater than that of the standard sequence.

Knibb (1982) collated the frequencies of the four cosmopolitan inversions in North American, Australasian and Japanese populations. Inversion frequency clines were found for all of the four inversions. In each region inversion frequency decreases with increasing latitude, although the precise relationship between frequency and latitude varies. In temperate latitudes, i.e. above 40^{o}, the frequency of each of these inversions is lower than 10%. In tropical and subtropical regions, the frequencies are generally greater than 20% and sometimes exceed 50%. The data indicate that inversions tend to be at higher frequencies for comparable latitudes in Japan than in either Australasia or North America (Inoue and Watanabe, 1979). The partial correlation and multiple regression coefficients of inversion frequencies on latitude and longitude showed that in all but one case there were negative and significant relationships between inversion

frequency and latitude. Even for the exception, *In(2R)NS*, the coefficients are negative and the partial correlation coefficient is close to statistical significance (Knibb, 1982). There are no significant differences either between inversions or between continents in the slope of the latitudinal clines. The data did not show any consistent relationship between inversion frequency and climatic factors in the three regions (Knibb *et al.*, 1981; Knibb, 1982).

Explanations for the latitudinal clines in both hemispheres invoking only random processes can be discounted because of the similar directions and immense geographic scale of the clines. It also seems unlikely that the observed distribution could reflect the spread of inversions which arose equatorially but are selectively advantageous throughout the species range, since the same directions of inversion clines were observed in North America (Voelker *et al.*, 1977) and Australia (Knibb, 1982) in which the patterns of colonization by *Drosophila melanogaster* differ. The similarity in the clines in three regions with very different histories of settlement and agricultural practices has been interpreted as convincing evidence for some kind of latitudinally varying selection pressure (Knibb 1982).

Latitudinal clines for allozyme frequencies of *Drosophila melanogaster* populations were first reported at the alcohol dehydrogenase (*Adh*) locus in North America (Pipkin *et al.*, 1973; Vigue and Johnson, 1973). Surveys of allozyme frequencies were extended to more loci and more geographic regions. Singh *et al.*, (1982) studied allozyme frequencies at 26 gene loci in nine populations of *Drosophila melanogaster* from five different continents. Seven loci (Glucose-6-phosphate dehydrogenase (*G-6pd*), Adh, Aldehydeoxidase (*Ao*), Esterase-C (*Est-C*), Esterase-6 (*Est-6*), Leucine aminopeptidase-D (*Lap-*

D) and Octanol dehydrogenase (*Odh*)) showed parallel north-south patterns in allozyme frequencies which varied latitudinally. Oakeshott *et al.*, (1981, 1982, 1983a,b) collated allozyme frequencies from natural populations sampled in North America , Asia, and Europe with their data for Australasia. They found that five allozyme loci (*Adh, G6pd,* 6-phosphogluconate dehydrogenase (*Pgd), Odh and Est-6*) showed significant latitudinal clines in all of these geographic regions. Later Singh and Rhomberg (1987) surveyed 117 gene loci in 15 populations of *Drosophila melanogaster* originating from five different continents. Among 117 gene loci (coding for 79 enzymes and 38 abundant proteins) they found 61 gene loci were polymorphic and 18 polymorphic gene loci showed latitudinal variation in allele frequency in America, Europe, Africa and the Far East.

Linkage disequilibria between inversions and allozyme loci located on the same chromosome arm have been found. A careful analysis of the allozyme and inversion data from populations along the east coast of the United States (Voelker *et al.*, 1978) indicated that although the inversion clines could enhance the allozyme clines, the inversions were only partly responsible. They estimated the contribution of *In(2L)t* to be 34% and 23% for the linked allozyme clines of α-Glycerophosphate dehydrogenase (α-$Gpdh^F$) and Adh^S respectively. They concluded that there would be clines of α-$Gpdh^F$ and Adh^S frequencies without an association with the inversion. They also found that the clines of $Est\text{-}6^S$ and Pgm^F were opposite to the contribution of the associated inversion *In(3L)P*, and these two clines would be steeper without the association with this inversion. The data also showed no consistent linkage disequilibrium between $Est\text{-}C^F$, Odh^F, $Acph^F$ (Acid phosphatase) and inversion *In(3R)P*. Similarly, Knibb (1983) concluded that inversion *In(2L)t* could only account for a small

fraction of the *Adh* latitudinal cline in Australia. Anderson *et al.*, (1987) extensively sampled populations over a large latitudinal transect in Australasia, and their analyses confirmed the result gained four years earlier that the clines in Adh^S frequency and *In(2L)t* were independent of each other.

Since the linked chromosome inversions fail to account for the allozyme clines, these clines may be the result of natural selection acting directly on the allozyme loci or on regions in linkage disequilibrium with the loci. A sensitive method in principle, to detect selection is to study the correlation between gene or genotype frequencies and particular environmental factors. Such a study often involves detailed biochemical and physiological investigations. This kind of study, aimed at identifying the selective factors which may maintain the allozyme clines, has been carried out. For example, Odh^S frequency decreases with increasing distance from the equator, and is positively related to the average daily maximum temperature for the hottest calendar month (Tmax) in Australasia and North America (Oakeshott *et al.*, 1983). But consistent seasonal variation in Odh^S was not observed in either continent (Cavener and Clegg 1981a,b; Franklin, 1981). Minawa and Birley (1978) found that Odh^S frequency had not diverged significantly among laboratory populations kept for over a year at different temperatures and on different food media, although the ODH-S allozyme has greater in vitro activity than ODH-F (Clarke, 1982). $Gpdh^F$ frequency decreases with increasing distance from the equator (Johnson and Schaffer, 1973; Anderson,1981), but there is no consistent relationship between the gene frequency and any climatic factor. The relative thermostabilities of GPD-F and GPD-S have little effect on the temperature tolerance of *Drosophila melanogaster* (Oakeshott *et al* 1982). The frequency of $G6pd^F$ is

positively correlated with latitude in Australasia, North America and Eurasia, but no consistent association between *G6pd*^{F} frequency and climatic variables has been found (Oakeshott,*et al.*, 1983) though G6PD-F is much less thermostable than G6PD-S (Bijlsma and van der Meulen-Bruijins, 1979). The frequency of *Pgd*^{F} showed a similar cline to that of the *G6pd*^{F} cline in Australasia and North America and was correlated with Tmax (Oakeshott *et al.*, 1983), but no difference in thermostability has been detected between PGD-F and PGD-S allozymes (Cavener and Clegg, 1981b). *Est-6*$^{1.00}$ frequency increases with the increasing latitude, but it was not related to any climatic variables (Oakeshott *et al.*, 1981). The directions of the *Est*$^{1.00}$ cline was opposite to the expectation based on the *in vitro* thermostabilities of the allozymes: the thermostability of the enzyme produced by *Est*$^{1.00}$ was greater than that produced by *Est*$^{1.10}$ (Danford and Beardmore, 1979).

Amongst the latitudinal clines of allozyme frequencies there are more data available for the *Adh* cline than for any of others. The cline was first reported in North American populations (Johnson and Schaffer, 1973; Smith *et al.*, 1984), and was then observed in Australasia (Wilks *et al.*,1980; Anderson,1981). Later Oakeshott *et al.*, (1982) collated the data on *Adh* frequencies from North America, Australasia, and Eurasia, and found a significant correlation between *Adh*^{F} and latitude; the frequency of *Adh*^{F} increases with increasing latitude. More recently, David *et al.*,(1986) observed a similar tendency between tropical African and European populations, and also in the southern hemisphere in populations between the equator and southern Africa. Using their original observations and selected published data from various parts of the world (Africa, Australia, Europe, west coast of the United States and countries around the

Mediterranean sea) David *et al.*(1988), found an overall increase of Adh^F with increasing latitude, but the relationship was not simply linear. The relationship varied in three geographic regions: tropical populations (between 0° and 30° latitude) are generally similar having a low frequency of Adh^F (average 15%) and a smooth increase with latitude; populations in a Mediterranean climate (between 30° and 42°) are all similar with much higher average Adh^F frequency (70%), a steeper slope and a broader range of variability at the same latitude, this phenomenon was called Mediterranean instability; cool temperate populations (above 42°, in France) have very high Adh^F frequency (95%).

The *Adh* cline has been claimed as strong evidence for latitude-related selection. A number of possible selective factors for the *Adh* cline have been carefully studied, and include differences in thermostability, temperature and alcohol tolerances between Adh^F and Adh^S lines. Often the biochemical and physiological properties were correlated with the cline, but further investigation in laboratory or natural populations failed to confirm any consistent selective factor (for details see discussion in Chapter 2). Variation in ADH activity may be related to some environmental gradients. In general the Adh^F allele is associated with high activity, the Adh^S allele with low activity and intermediate activity with the Adh^{FChD} allele (Rasmuson *et al.*, 1966; Gibson, 1970; Gibson, 1972; Vigue and Jonhson, 1973; Lewis and Gibson, 1978; Maroni,1978; McDonald *et al.*,1980). Differences in ADH activity between lines of the same genotype were found to be due to a series of linked and unlinked modifier loci (Ward and Hebert,1972; Thompson *et al.*, 1977; McDonald and Ayala, 1978; Laurie-Ahlberg *et al.*, 1980; Birley *et al.*, 1980; Maroni *et al.*, 1982; Maroni and Laurie-Ahlberg, 1983). Environmental factors also affect

the level of ADH activity. For example Clarke *et al.*(1979) have shown that the amount of yeast in the media greatly changes the amount of ADH protein (a fourfold increase) and the exposure of eggs, larvae and embryos to ethanol, induced higher ADH activity (Horikawa *et al.*,1967; Gibson, 1970; Bijlsma-Meeles, 1979). Gibson and Wilks (1988) found that populations breeding in a winery, where the concentration of ethanol in breeding site was higher than at sites outside the winery, had a higher level of ADH activity.

Since previous work has not revealed any consistent selective factor responsible for the *Adh* cline, it must be considered a possibility that the selection may not act on the gene itself, but on factors which are linked to the gene. This possibility could be tested by surveying the molecular variation in the region around the *Adh* structural gene to see if there is any linkage disequilibrium between the neighbouring molecular variation and *Adh* alleles, and investigating whether any of the variation shows a parallel latitudinal cline with that of Adh^F frequency. For example, restriction endonuclease variants closely linked to the structural gene may be under environmental selection instead of the *Adh* gene itself.

Restriction map variation, which includes insertion/deletion and restriction endonuclease site recognition variation, in the nuclear genome of *Drosophila melanogaster* has been extensively studied. For example Aquadro *et al.*, (1986) found that about 80% of the 48 lines from eastern United States populations of *Drosophila melanogaster* carry one or more of the total of 16 types of insertions/deletions in the 13kb *Adh* gene region. Some were identified as mobile elements, and the expression of the *Adh* gene was affected by some of these

insertions. Strong non-random associations were found among some of the variants.

In my study I first aimed to survey the geographic patterns of allozyme variations in the Chinese populations, as little was known about allozyme frequency in populations of *Drosophila melanogaster* from mainland China. Will any allozyme frequencies show latitudinal clines in China as they do in other continents? If there are clines, will the direction of the clines in China be the same as that in other continents? If they are, then these data will support the argument that a consistent association between gene frequency and latitude in different zoogeographic zones indicates the existence of natural selection. Eight Chinese *Drosophila melanogaster* populations were sampled at latitudes comparable to those of Australia, and allozyme frequencies at eight polymorphic loci were investigated in order to test the generality of the latitudinal clines of allozyme frequency in Australia and China. This work is described in Chapter 2.

My second aim is to investigate whether any factor linked with the *Adh* gene is responsible for the *Adh* cline. Restriction endonuclease variation in the 12kb region encompassing the *Adh* gene has been investigated in the Australian and Chinese populations to see if there is any restriction endonuclease variant which shows a latitudinal cline parallel to the *Adh* cline. A comparison of the restriction map variation between Australian and Chinese populations of *Drosophila melanogaster* might also show differences in the history of colonization by this species in the two countries, in the amount of variation between *Adh* genotypes. This work is described in Chapter 3. The identification of the insertions found in the investigation is described in chapter 5.

My third main aim is to investigate ADH activity along the *Adh* frequency cline in the two countries to see if it is the same at different latitudes. Do the ADH activity levels show a latitudinal cline? Does the level of ADH-F or ADH-S alleloenzymes vary between different geographic sites? Is this variation correlated with latitude? Are there differences in ADH activity between Australian and Chinese populations? In order to answer these questions I have assayed ADH activity in six Chinese and seven Australian populations, and this work is described in Chapter 6.

Table 1.1 Genetic variation showing geographic differentiation or latitudinal clines in natural populations of *Drosophila melanogaster*.

Trait	Geographic pattern	Authors
body weight, wing length, thorax length, sternopleural chaetae number, abdominal chaetae number, ovariole number	The parameters of the six morphological traits are always larger in European flies than in African flies. Male and female fresh weight and ovariole number increase with the increasing latitude.	David and Bocquet (1975a,b) David (1979)
oviposition rhythm	The height of the oviposition peak decreases with increasing latitude.	Allemand and David (1976) David (1983)
light sensitivity	An increase in positive phototaxis from Tropical African to European populations.	Medioni (1958)
sexual activity	African males were sexually more active than French males, the French females were more receptive to males than those from Afrotropical populations.	Cohet and David (1980)

Trait	Geographic pattern	Authors
cuticular hydrocarbons	Flies from Europe, North America, and North Africa possess significantly higher levels of 7-tricosene and 7,11-heptacosadiene in females and 7-tricosene in males than populations from south of the Sahara.	Antony and Jallon (1982); Jallon (1984) Antony et al., (1985) Jallon and David (1987)
egg production	African flies have a lower fecundity than European flies.	David (1970); Pianka (1970); Bouletreau-Merle et al., 1982);
alcohol tolerance	Alcohol tolerance decreases from south to north in Australian populations, and from European to tropical African populations.	David and Bocquet (1975b) Parsons (1980a)
temperature tolerant	Temperate flies were more tolerant to cold and less tolerant to heat than tropical ones.	Cohet (1980); David (1983)
desiccation	Tropical populations were less tolerant to high temperature, desiccation and cold stress than a temperate population in Australia.	Parson, (1977, 1980a,b)

Trait	Geographic pattern	Authors
perpetuation of the sigma virus	The frequency of virus type II decreases with increasing distance from France.	L'Heritier (1970); Fleuriet (1976, 1980, 1986)
P element	The frequency of P element gradually decreases from western Europe to middle Asia and from the Far East to middle Asia. Along the east coast of Australian population the frequency of P elements decreases from north to south.	Anxolabehere et al. (1985); Boussy (1987); Boussy and Kidwell (1987) David and Capy (1988).
mitochondrial	mtDNA show high level of local differentiation in populations from North America, Europe, Africa, South African, Australia and Asia.	Hale and Singh (1987)
chromosomal inversions	The frequencies of four cosmopolitan inversions increase with decreasing latitude in North American, Australasian and Japanese populations.	Knibb (1982, 1983)

Trait	Geographic pattern	Authors
allozyme frequency	At least five allozyme loci show significant latitudinal clines in populations from North America, Asia, Europe and Australia.	Oakeshott, et al., (1981, 1982,1983a,b) Singh, et al. (1982); Singh and Rhomberg (1987)

CHAPTER 2

ALLOZYME FREQUENCIES IN CHINA: COMPARISON WITH PATTERNS ON OTHER CONTINENTS

Chapter 2: Allozyme frequencies in China: comparison with patterns on other continents

2.1 Introduction

As the consistent latitudinal clines of allozyme frequencies over a large geographic range in both hemispheres, and across different zoogeographic zones, could be interpreted as convincing evidence for natural selection in the selection-neutralist debate, it is important to test for the consistency of such allozyme frequency clines. Numerous studies have been made to survey allozyme frequencies in natural populations of *D.melanogaster*. Oakeshott *et al.*, (1981, 1982, 1983 a,b) collated allozyme frequencies from north American, Asian, European and Australasian populations of *D.melanogaster* and analysed their partial correlations and multiple regressions with latitude, longitude and climatic factors. They found six allozyme loci showed latitudinal clines in Australian populations, five of which also were present on other continents (table 2.1).

In the collated analyses of allozyme frequencies, data from Asian populations were very limited. Except for the data from several west Asian localities (Israel and U.S.S.R.) data for the collated analyses of *Adh, Gpdh, Odh and Acph* frequencies were only available for populations at three Japanese locations. For the collated analyses of *Est-6, Pgm and G6pd* data only one Japanese location was available.

Watada *et al.*(1986) investigated allozyme frequencies in populations of *D.melanogaster and D.simulans* from 28 locations in the Japanese islands. Data from populations of *D.melanogaster* in 18 locations showed that there was a higher Adh^S frequency in populations of the southern and central islands, although statistical analyses were not carried out. *Gpdh, Est-6 and G6pd* were the other three loci

studied and their allele frequencies varied from population to population, but did not show latitudinal clines. *Odh* showed a low level of polymorphism, and *Acph* was found to be monomorphic.

Singh *et al.*(1982) studied the genetic variation in nine natural populations of *D.melanogaster* from different continents. In their study samples from two East Asian populations, Taiwan and Vietnam, were included. The allozyme frequencies of the two subtropical (Taiwan) and tropical (Vietnam) populations were similar to those from the temperate populations of north America and Europe. For example, in comparison with other tropical populations, these two populations showed much higher Adh^F frequencies(0.95 and 1.00). The data suggested that in general, the clinal patterns which are seen in North America, Europe and Africa seem not to be repeated in the East Asian populations.

In order to test the generality of the latitudinal differentiation found in north America, Europe and Australia, more data from Asia, especially from East Asia is necessary. The colonization of Asia by *Drosophila melanogaster* is supposed to have occurred at a similar time to Europe, much earlier than in North America and Australia (David and Capy, 1988). *D. melanogaster* only became established in Australia after European settlement. Similarly, the evidence from museum collections shows that *Drosophila melanogaster* became established in North America in the second half of the nineteenth century (David and Capy, 1988). Such a difference in history could cause different patterns of geographic differentiation.

Drosophila melanogaster occurs in the People's Republic of China(P.R.C.) from 20° to 50° N, which is similar to the latitude of Australia(10° to 45° S). In the present study, allozyme frequencies at eight loci in eight *Drosophila melanogaster* populations from east

P.R.C. were scored and collated with data from recent observation on Japanese populations (Watada *et al.* 1986; see table 2.2.2).

2.2 Materials and Methods

2.2.1. Sample collection and maintenance

Adult *Drosophila melanogaster* were collected at eight sites in the eastern region of the People's Republic of China (P.R.C.) over a latitudinal range of 20°N (Fig. 2.1). The populations, which were all large, were sampled in the autumn of 1986 at fruit stores and wineries in eight cities and the samples were air freighted to Canberra where at least 100 single female lines were established from each population and maintained by mass transfer at 21±1° C on standard culture medium : 10g agar, 50g glucose, 50g maize meal, 26g sucrose, 22.5g wheat germ, and 6g yeast in 1 liter of water with 12.5 ml of acid mix (42% propionic acid, 4% orthophosphoric acid) (Gibson and Wilks, 1988). 220-430 genes were scored for each populations.

2.2.2. Electrophoresis conditions and stains:

Electrophoresis was carried out on cellulose acetate membranes (Chemetron), the buffers used and the conditions were mostly based on published protocols with some modifications (see below). Before electrophoresis the membranes were soaked in the running buffer for at least 20 minutes, then blotted twice between two sheets of blotting paper. Single fly homogenates were transferred to the membrane by a plastic comb. After electrophoresis the membrane was stained by putting a piece of blotting paper soaked in staining buffer underneath the membrane, which was then placed in a plastic box and kept in a dark cupboard until the stain intensity was satisfactory. The

electrophoresis conditions and stain solutions for each allozyme system were as follows:

1.alcohol dehydrogenase (ADH; EC 1.1.1.1.) (follows Lewis and Gibson, 1978)

Electrophoresis at 300V. for 30 min. Running buffer: 70mM Tris, 5mM boric acid, 7mM EDTA PH8.8. Staining buffer: 50mM orthophosphate buffer pH 7.8, 1% isopropanol, 0.5 mg/ml NAD, 0.01mg/ml phenazine methosulphate (PMS), 0.5mg/ml tetrazolium salt (MTT). Stain for 3-5 min.

Thermostable ADH variants (Adh^{FChD}) were detected by following the standard electrophoresis procedure and then sealing the membrane in transparent plastic wrapping film (Glad-wrap) and immersing it in a 43°C water bath for 15 seconds prior to activity staining. The electrophoretic pattern of Adh^{FChD} after heat treatment and the control without heat treatment were as shown in Figure 2.2 (Wilks *et al.*, 1980).

2. α-glycerophosphate dehydrogenase (GPD; EC 1.1.1.8.) (follows Gibson *et al.*, 1986)

Electrophoresis at 450V. for 45 min. Running buffer: 126 mM Tris, 42mM citric acid, 9.26mM EDTA pH 6.6. Staining buffer: 100mM Tris-HCl pH 8.6 containing 7mg/ml D,L α-glycerophosphate, 2mg/ml NAD, 0.5mg/ml MTT, 0.01mg/ml PMS, 2mg/ml EDTA. Stain for 3-5 min.

3. esterase-6 (EST-6; EC 3.1.1.1.) (follows P.H. Cook *et al.*, 1987)

Electrophoresis at 250V, 4°C, for 110 min. Running buffer: 80mM Tris-borate, 1.3mM EDTA pH 8.5. Staining buffer: 0.1M phosphate pH 6.0, 10mg α,β-naphyl acetate (in acetone), 10mg Fast garnet GBC salt. Stain for 10-15 min. Destainned in 15% ethanol and 5% glacial acetic acid.

4. phosphoglucomutase (PGM; EC 2.7.5.1.) (follows Shaw and Prasad, 1970)

Electrophoresis at 350V. for 30 min. Running buffer 80mM tris-borate PH 8.9, 1.5mM EDTA. Staining buffer: 0.1M Tris-HCl pH 8.0, 0.5mg/ml MTT, 0.01mg/ml PMS, 50ng/ml EDTA, 0.05M Mgcl, 10mg Sodium glucose-1-phosphate, 20 units glucose-6-phosphate dehydrogenase, 10 mg NADP. Stain for 5 min.

5. octanol dehydrogenase (ODH; EC 1.1.1.73.) (follows Courtright *et al.*, 1966)

Electrophoresis at 350V. for 45min. Running buffer: as EST-6. Staining buffer: 1ml octanol, 15mg Nitro blue tetrazolium (NBT), 15mg NAD+ in 10ml of 1.0M Tris-HCl buffer, pH 8.5. After staining for one hour 4mg PMS was added.

6. acid phosphatase (ACP; EC 3.1.3.2.) (follows Ayala *et al.*, 1972)

Electrophoresis at 400V. for 45 min. Running buffer as EST-6. After electrophoresis the gel was dipped in 0.5M boric acid for 30min to adjust the pH, and then stained in 0.1M Tris-Maleat buffer, pH 5.3, with 1mg/ml α-naphthyl acidphosphate and 1mg/ml Fast black K salt, for 1 hour.

7. glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49.) (follows Bijlsma and Van Delden, 1977).

Electrophoresis at 350V. for 45 min. Running buffer: 0.15M Tris-citric acid, pH 7.5 with 5mM EDTA. Staining buffer: 0.1M Tris-HCl, pH 7.5, 10 mM MgCl, 10mg NADP, 0.5mM MTT, 10mg Glucose-6-phosphatase, 2mg PMS. Stain for 5-10 min.

8. 6-phosphogluconate dehydrogenase (PGD; EC 1.1.1.43.) (follows Bijlsma and Van Delden, 1977)

Electrophoresis at 350V. for 45min. Running buffer and staining as G-6PD except the substrate was sodium 6-phosphogluconate instead of glucose-6-phosphate.

2.2.3. Climate data

Longitude, latitude and the 30 year averages of climate data from a weather station at each collecting site were obtained from Zhang and Lin (1985) and Sheng (1986), (see table 2.2.1). The altitudes of the collecting sites were all below 100 meters. The following four variables were calculated:

Maximum temperature (Tmax): average daily maximum in ^{o}C for the hottest calendar month at the station.

Minimum temperature (Tmin): average daily minimum in ^{o}C for the coldest calendar month at the station.

Maximum rainfall (Rmax): total rainfall in millimeters for the wettest calendar month at the station, square root transformed for the analyses.

Minimum rainfall (Rmin): total rainfall in millimeters for the driest calendar month at the station, square root transformed for the analyses.

The relationship between allele frequencies and latitude, longitude and the climatic variables were examined by partial correlation and multiple regression analyses using computing programs from the SPSS package (Nie *et al.*, 1975).

Data on allele frequencies at some of the loci were available for eighteen localities in Japan (Watada, Tobari and Ohba; 1986) and these data for sites on each of the six islands (see Table 2.2.2) were collated with the data collected from the P.R.C. sites for the

analyses of partial correlation and multiple regression with latitude and longitude.

2.3.Results

The alleles detected and their frequencies at the eight loci scored in the eight P.R.C. populations are given in table 2.3.1.

At four loci, *Pgm, Odh, Acph and Pgd*, very little geographic variation was observed, with the frequency of the most common allele always greater than 0.9. For *Pgm* the frequencies of the commonest allele $Pgm^{1.00}$ were around 0.95 in most populations; for the *Odh* gene Odh^{F} was fixed in seven populations; $Acph^{F}$ and Pgd^{F} were fixed in five populations. None of the alleles at these loci showed any significant variation with latitude or longitude.

Gpdh was polymorphic in each of the populations sampled with two or three alleles. The frequencies of $Gpdh^{F}$ varied from 0.30 to 0.81, but was not significantly correlated with latitude or longitude, either in the P.R.C. samples or in the collated data set. One population, Xiamen, had a relatively high frequency of $Gpdh^{UF}$, which has only been found in previous surveys as a rare allele.

G6pd was polymorphic in six populations with the commonest allele, $G6pd^{F}$, significantly correlated with distance from the equator in the partial correlation (and also in the total data set) but not in the multiple regression coefficient (table 2.3.2.). The frequency of this allozyme showed a shallow latitudinal cline: with higher frequencies of $G6pd^{F}$ in north China.

The *Est-6* and *Adh* loci were polymorphic in each population sampled, the frequencies of Adh^{F} varied from 0.39 in a population from southern China (Guangzhou) to 0.86 in a population from northern China (Luda, also known as Dalian.). The variation in the two allozyme

frequencies was also significantly correlated with latitude, both in the P.R.C. samples and in the collated data (Table 2.3.2.). *Est-6*$^{1.00}$ decreased in frequency with increasing distance from the equator, but this was opposite in direction to the differentiation previously reported in populations from North America (Johnson and Schaffer, 1973), or from Europe and Australasia (Oakeshott *et al.*,1981).

At the *Adh* locus the variation was consistent with previous studies in showing that *Adh*^{S} frequency decreases with increasing latitude (Vigue and Johnson,1973; Wilks *et al.*, 1980). However a surprising feature of the *Adh* data is the high frequency (up to 36%) of heat resistant *Adh*^{F} alleles in most southern P.R.C. populations (a similar result has been obtained in other samples from this region by L. Chai, personal communication). The biochemical properties of these heat resistant alleles was investigated further and the results are described in chapter 6.

The allozyme frequency data showed that geographic differentiation occurs between Chinese populations of *Drosophila melanogaster*, in particular the latitudinal clines of *Adh*^{S}, *Est*$^{1.00}$ *and G6pd*^{F} indicate that the north-south differential pattern also occurs in China.

In the data from the eight P.R.C. populations, none of the allele frequencies at any locus were significantly correlated with any of the climatic variables after the latitudinal variation had been taken into account (table 2.3.3.)

2.4 Discussion

The landmasses of the People's Republic of China and Australia span similar latitudes but lie in different hemispheres, so that *D. melanogaster* sampled from the two areas provides tests of the generality of latitudinal differentiation of specific polymorphic

loci. Previous surveys, mainly of populations in Australia, Europe and Northern America, have shown that five loci, *Adh, Odh, G6pd, Est-6 and Pgd* exhibit latitudinal variation, although the degree of association varies between continents. Previous work (Oakeshott *et al.*, 1983 a, b) found the average frequency of Pgd^F in Europe/Asia to be 0.99, and this allele is nearly fixed in all the populations sampled from this area, except for one population, where the Pgd^F frequency was 0.8. Thus meaningful tests for clines within this area were precluded. Similarly, low levels of polymorphism occurred for *Odh and Acph*: the average frequencies of Odh^S *and* $Acph^S$ were: 0.046 and 0.017, 0.10 and 0.054, 0.026 and 0.013 in Australasia, Northern America and Europe/Asia respectively, nevertheless statistical analyses of a large collated data set indicated that they showed significant latitudinal clines in some continents (Oakeshott *et al.*, 1983).

In the present study, the first to sample populations in mainland China, four loci, *Pgd, Acph, Odh and Pgm* showed quite low levels of polymorphism, which is similar to the data collected from Japan and to the results of previous surveys in other continents, especially in Europe/Asia (Oakeshott *et al.*, 1983a.b.). For example, in the P.R.C. and Japanese data set the highest frequency of Odh^S is 0.23, but in most populations it is less than 0.05, the average frequency of Odh^F is 0.95, and the average frequency of $Acph^F$ is 0.98. The highest frequency of $Acph^S$ is 0.08, and $Acph^F$ is fixed in two thirds of the populations from these areas. Tests of latitudinal variation for loci exhibiting little variation were not calculated.

The tendency of alleles at certain loci to be fixed in a large proportion of populations suggest that these loci may be under purifying selection in these areas.

The inconsistent result for *Est-6*, compared with earlier surveys, is most surprising as this locus is polymorphic in most populations sampled. As the pattern of variation was in the opposite direction to that previously found in the Northern Hemisphere I paid particular attention to the electrophoresis conditions I had used. The conditions used in this study were chosen because they had been shown to maximise the discrimination of *Est-6* phenotypes (Cook *et al.*, 1987). In addition the same control alleles as used by Cook *et al.* were used in my experiments. Thus any imprecise resolution of *Est-6* phenotypes is unlikely to have occurred in my study (P. Cook, personal communication). There are a number of possible explanations for the result. The variation in $Est\text{-}6^{1.00}$ in the P.R.C. populations might be due to linkage disequilibrium with the inversion *In(3L)P*, which has breakpoints on either side of the locus and which decreases in frequency with increasing latitude in North America (Voelker *et al.*,1977) and in Australasia and Europe (Knibb, Oakeshott and Gibson,1982). Cytological investigation of the P.R.C. and Japanese material will test this possibility. It may be that variation tracks some thing which does not vary latitudinally in P.R.C. The relatively low temperatures during the collecting time (November) may have exaggerated the $Est\text{-}6^{1.00}$ frequency in the southern China populations. Franklin (1981) monitored six populations over five years in the Hunter Valley, Australia and found that $Est\text{-}6^{1.00}$ frequency consistently declined in the hot summer months and rose again in winter. In 1971 the frequency of $Est\text{-}6^{1.00}$ in the population of the Hunter Valley dropped from 0.95 (in winter) to 0.67 (in summer).

It is also relevant to the inconsistency of the variation in $Est\text{-}6^{1.00}$ that the frequency of the allele does not change in the direction expected when populations are transplanted and reared in

different latitudes (Oakeshott *et al.*, 1988). It is also possible that samples scored by others contained some misclassification.

There are some data indicating a relationship between the properties of the EST-6 allozyme, temperature and fitness. Biochemical studies have indicated that the thermostability of the enzyme produced by $Est\text{-}6^{1.00}$ is greater than that produced by $Est\text{-}6^{1.10}$ (Danford and Beardmore, 1979). Laboratory perturbation experiments and fitness component analyses carried out between 25° and 30°C indicated higher equilibrium $Est\text{-}6^{1.00}$ frequency at these temperatures than between 18° and 20° C (MacIntyre and Wright, 1966). However these results from laboratory experiments are not in accord with the population distributions of $Est\text{-}6^{1.00}$ frequencies in natural populations in North America, Europe and Australasia. *Est-6* P.R.C. data remain difficult to reconcile with those collected elsewhere, but they do show that the direction of the relationship with latitude is not consistent as had previously been thought to be the case. The data argue against the idea that the geographic pattern in *Est-6* is caused by environmental varying factors which act directly on the *Est-6* phenotype.

The data for *G6pd* are consistent with those from other continents in showing a relatively shallow latitudinal cline. The partial correlation coefficients of $G6pd^F$ frequencies on latitude were 0.44, 0.32 and 0.67 in Australasia, Northern America and Europe/Asia respectively. The mean $G6pd^F$ frequencies differ between continents e.g.: 0.59 (Australasia), 0.18 (North America) and 0.95 (Europe) (Oakeshott *et al.*,1983). The data from P.R.C. and Japan showed similar high $G6pd^F$ frequencies and shallow latitudinal clines to those found in Europe. The variation in the present data was not related to any of the climatic variables and again this mirrored earlier results which

showed that there were no consistent climatic relationships in data from other continents (Oakeshott *et al.*, 1983a).

The latitudinal differentiation in Adh^S in the P.R.C. populations is consistent in form with that previously found in Australasia (Wilks *et al.*, 1980; Anderson, 1981; Oakeshott *et al.*, 1982). In samples collected in 1986 the pattern was unchanged from the first studies carried out in 1978, (J.B. Gibson and C. Jiang, unpublished data and see Anderson *et al.*,1987). In general Adh^S frequencies are lower in the P.R.C. population than in populations at comparable latitudes in Australia but otherwise the latitudinal differentiation is similar. In earlier surveys (Oakeshott *et al.*, 1982) the frequency of Adh^S was related to Tmax in populations in North America, and to Rmax in North America and Australasia. But later surveys failed to find this association (Anderson *et al.*,1987). In the P.C.R. data the frequency of Adh^S was not correlated with any of the four climatic variables.

Recent population samples from Eastern and Western regions of North America have shown that the latitudinal cline in Adh^S is now less marked in the eastern region (Gail Simmons, personal communication) and in the west region the relationship between Adh^F frequency and latitude, although positive, is weak and non-significant (p=0.46) (Smith *et al.*,1984). They found the distribution of *Adh* alleles on the west coast of the United States is complex, with clines in California (P=0.01, significant in statistical analysis) but non-significant in association with latitude (p=0.12) in Oregon, Washington and British Columbia. The Adh^S frequency and relationship to latitude (linear regression) in the west coast of the United States is shown in Figure 2.3. The failure to observe a cline in *Adh* frequency might be due to the small number of populations they sampled and to the relatively narrow latitudinal region covered in the

study. In the western part of the U.S.A. it may also be due to altitudinal variation modifying the climate. Singh *et al.*, (1987) explained that their failure to recognize some allozyme latitudinal variation that does in fact exist was partly due to the small number of localities they sampled.

Temperature has been suggested as a selective factor acting on *Adh* cline. Biochemical studies (Chambers *et al.*,1984) indicated that thermostabilities of Adh^F *and* Adh^S were nearly the same *in vitro. In vivo* studies by van Delden and Kamping (1980), Vigue and Sofer (1976) and Gionfriddo *et al.*, (1979), found no relationship between heat stability of *Adh* products and adult mortality when temperature of 35°C and 17°C to 29°C were used. Vigue *et al.*,(1982) studied the effect of a combination of ethanol and temperature on the competitive success of Adh^F *and* Adh^S in *D.melanogaster* larvae and on the ADH activity of adults and larvae subjected to various temperatures (17, 25 and 30°C). They found that after 10 generations, the frequency of Adh^F increased at each temperature, but controls with no ethanol showed no change in the frequencies of Adh^F. Gionfriddo and vigue (1978), Gionfriddo *et al.*, (1979) and Franklin (1981) also found no correlation between seasonal variation in temperature and seasonal variation in the frequency of Adh^F. Temperature seems unlikely to be the selective factor maintaining the latitudinal cline in Adh^S frequency.

An unexpected feature of the P.R.C. data is the high frequency of thermostable Adh^F variants which share characteristics with ADH-F.CHD (Gibson *et al.*, 1980). Similar *Adh* variants have previously been found in surveys of natural populations in Northern America (Sampsell, 1977, Smith *et al.*, 1984) and Australia (Wilks *et al.*, 1980; Gibson, Wilks and Chambers,1982) at frequencies as high as 0.16 in one population, but generally with average values closer to 0.04. These surveys show

that populations in regions of Australasia and North America with climates similar to that of Southern China do not have frequencies of Adh^{FChD} above 0.03. A heat resistant Adh^{F} allele has also been found at low frequency (less than 0.01) in the Canary Islands (Hernandez *et al.*, 1986) but not in central African populations (J. David, personal communication). It is relevant that Adh^{F} is relatively rare in tropical Africa (David, 1982) where, on the basis of biogeographical and phylogenetic evidence, it is believed *D. melanogaster* originated (Lemeunier *et al.*, 1986; David and Capy 1988).

The evolutionary history of *D.melanogaster* and the worldwide distribution of Adh^{FChD}, which probably arose from a mutation in Adh-F, (Gibson, Wilks and chambers, 1981) prompts the speculation that the Adh^{FChD} mutation occurred in southern China and spread, possibly in association with human migration, to North America and Australasia in the nineteenth century. Although many introductions of *D.melanogaster* from different countries are likely to have occurred in both North America and Australia, Guangzhou (Canton) was a major trading port and there is historical evidence (Huck, 1968) that Chinese migrants to N. America and Australia in the mid-nineteenth century came mainly from the southern provinces of Guangdong of which Guangzhou is the capital, and Fujian, of which Xiamen and Fuzhou are the two largest cities. The ships that transported the migrants carried fruit, vegetables and fermented liquor from the port of embarkation and it is possible that samples of *D. melanogaster* accidently transported from Guangzhou were likely to have contained Adh^{S}, Adh^{F} and Adh^{FChD} alleles at similar frequencies and could have given rise to the widespread distribution of Adh^{FChD}.

If Northern America and Australia were only recently colonised by *D.melanogaster* then the latitudinal variation in Adh^{S} (and to a

lesser extent that in *G6pd*^{F}) on these continents has developed over the past century in contrast to mainland China where *D. melanogaster* is likely to have been endemic for much longer. Singh and Rhomberg (1987) have discussed two selective and two non-selective mechanisms which could give rise to latitudinal variation. For example allele frequencies might be tracking latitudinally varying environmental factors or there may be gene flow between two distinct latitudinally separate selective regimes. The non-selective causes include the intermixing of differentiated populations and gradients in allele frequencies deriving from change in seasonal cycles along a latitudinal gradient. Notwithstanding the speculation about human mediated dispersion of *Adh*^{FChD}, the consistent *Adh*^{S} latitudinal cline, at least in eastern Australia and eastern China, coupled with evidence that transplanted populations change in *Adh* frequency towards the recipient area (Oakeshott *et al.*, 1988), is strongly suggestive of some kind of balancing selection focussed on phenotypes to which variation in the *Adh* region of the genome makes a significant contribution.

Table 2.1 Partial correlation coefficients for allozyme allele frequencies with latitude in *Drosophila melanogaster* populations, data were taken from Oakeshott et al.(1981, 1982, 1983a,b).

*p<0.05, **p<o.o1, ***p<0.001

Continent	*Adh*^{S}	*Gpdh*^{F}	*Est-6*$^{1.00}$	*G6pd*^{F}	*Pgd*^{F}	*Odh*^{S}	*Acph*^{S}
Australia	$-.75^{***}$	-.23	$.28^{*}$	$.44^{**}$	$.63^{***}$	$-.59^{**}$	$-.49^{*}$
N. American	$-.48^{***}$	$-.57^{***}$	$.31^{*}$	$.32^{*}$	$.84^{***}$	$-.54^{**}$	.04
Asia	$-.58^{***}$						
Eur/Asia			$.53^{**}$	$.47^{*}$		$-.66^{**}$	-.16

Table 2.2.2 Averages of allozyme frequencies in populations of *D. melanogaster* sampled from Japanese islands.

Data taken from Wadata et al, 1986. n = number of genes sampled.

		HOKAIDO	HONSHU	SHIKOKU	KYSHU	RYNKYN	OGASAWARA
Allele	n	43° N	36° N	33°42'N	32°N	27°N	27° 30'N
Adh^S	8776	0.20	0.27	0.13	0.50	0.79	0.61
Gpdh^F	8756	0.84	0.74	0.73	0.72	0.89	0.78
Est-6^S	8382	0.88	0.85	0.92	0.95	0.60	0.60
Odh^F	8680	0.98	0.95	0.98	0.98	0.76	1.00
Acph^F	7060	0.92	1.00	0.99	1.00	1.00	1.00
G6pd^F	7842	0.76	0.86	0.93	0.96	0.69	1.00

Table 2.3.1. Allele frequencies in the eight P.R.C. populations. The number of genes scored for each locus is shown in parenthesis at each site and the latitude of the site is indicated.

Allele	HAIKOU 20° 02′N (220)	GUANGZHOU 23° 08′N (440)	XIAMEN 24° 27′N (330)	FUZHOU 26° 05′N (380)	SHANGHAI 31° 10′N (380)	XUZHOU 34° 17′N (430)	JINAN 37° N (390)	LUDA 38° 54′N (390)
Adh^{S}	0.43	0.25	0.21	0.23	0.20	0.20	0.17	0.10
Adh^{F}	0.43	0.39	0.64	0.66	0.80	0.80	0.76	0.86
$Adh^{F.Ch.D}$	0.14	0.36	0.15	0.10	–	–	0.06	0.04
$Gpdh^{UF}$	–	–	0.13	–	–	–	–	–
$Gpdh^{F}$	0.81	0.66	0.39	0.30	0.44	0.54	0.46	0.51
$Gpdh^{S}$	0.19	0.34	0.48	0.70	0.56	0.46	0.54	0.49
$Est\text{-}6^{1.00}$	0.96	0.96	0.98	0.72	0.76	0.61	0.28	0.34
$Est\text{-}6^{1.10}$	0.04	0.04	0.02	0.28	0.24	0.39	0.72	0.66
$G6pd^{F}$	0.86	0.94	0.92	0.80	0.98	1.00	0.97	1.00
$G6pd^{S}$	0.14	0.06	0.08	0.20	0.02	–	0.03	–
$Pgm^{1.20}$	0.05	0.05	0.10	0.06	–	0.08	–	0.06
$Pgm^{1.00}$	0.95	0.93	0.90	0.94	0.93	0.92	0.98	0.94
$Pgm^{0.70}$	–	0.02	–	–	0.07	–	0.02	–
Odh^{F}	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00
Odh^{S}	–	–	–	0.01	–	–	–	–
$Acph^{F}$	1.00	1.00	1.00	0.92	1.00	1.00	0.94	0.97
$Acph^{S}$	–	–	–	0.08	–	–	0.06	0.03
Pgd^{F}	1.00	0.97	1.00	0.97	0.95	1.00	1.00	1.00
Pgd^{S}	–	0.03	–	0.03	0.05	–	–	–

Table 2.3.2
Partial correlation (r) and multiple regression coefficients (b) of angularly transformed allele frequencies on latitude and longitude in (a) the P.R.C samples and (b) including data for Japanese populations

		r		b	
		lat	long	lat	long
Adh^S	(a)	-0.72*	-0.50	-0.62*	-0.34
	(b)	-0.64*	+0.53*	-0.70*	0.54*
$Est\text{-}6^{1.00}$	(a)	-0.94**	0.52	-1.06*	0.23
	(b)	-0.49*	0.3	-0.55	0.31
$G6pd^F$	(a)	0.65*	-0.06	0.73	0.48
	(b)	0.19	-0.23	0.22	-0.26

*p<0.05, **p<0.01.

* Partial correlations (r) of allele frequencies with latitude (longitude) were calculated while the effect of longitude (latitude) was controlled.

Table 2.3.3 Partial correlation (r) and multiple regression coefficients (b) of angularly transformed allele frequencies on latitude (lat.), longitude (long.), maximun and minimun temperature (Tmax, Tmin) and rainfall (Rmax, Rmin) in the P.R.C. populations.

	r						b					
	lat.	long.	Tmax	Tmin	Rmax	Rmin	lat.	long.	Tmax	Tmin	Rmax	Rmin
Adh^{S}	-.72*	-.50	.081	.41	.17	.11	-.82*	-.70*	.64	.81*	.72*	.39
Gpdh^{F}	.085	-.64	-.38	.52	-.029	-.37	-.46	-.73*	.10	.45	.52	-.057
Est-6$^{1.00}$	-.94**	.52	-.21	.76	-.29	.47	-.93**	-.37	.69*	.91**	.48	.81*
G6pd^{F}	.65*	-.056	-.017	-.20	-.20	-.10	.71*	.37	-.61	-.73	-.62	-.45

*P<0.05, **P<0.01

* Partial correlations (r) of allele frequencies with one variable were calculated while the effects of other five variables were controlled.

Figure 2.1 The locations of the eight P.R.C. populations. Also shown are the locations of the eighteen Japanese populations (Watada, *et al.*, 1986) which comprise one sample from Hokkaido, eleven samples from Honshu, one sample from Shikoku, one sample from Kyushu, three samples from Ryukyu and one sample from Ogasawara.

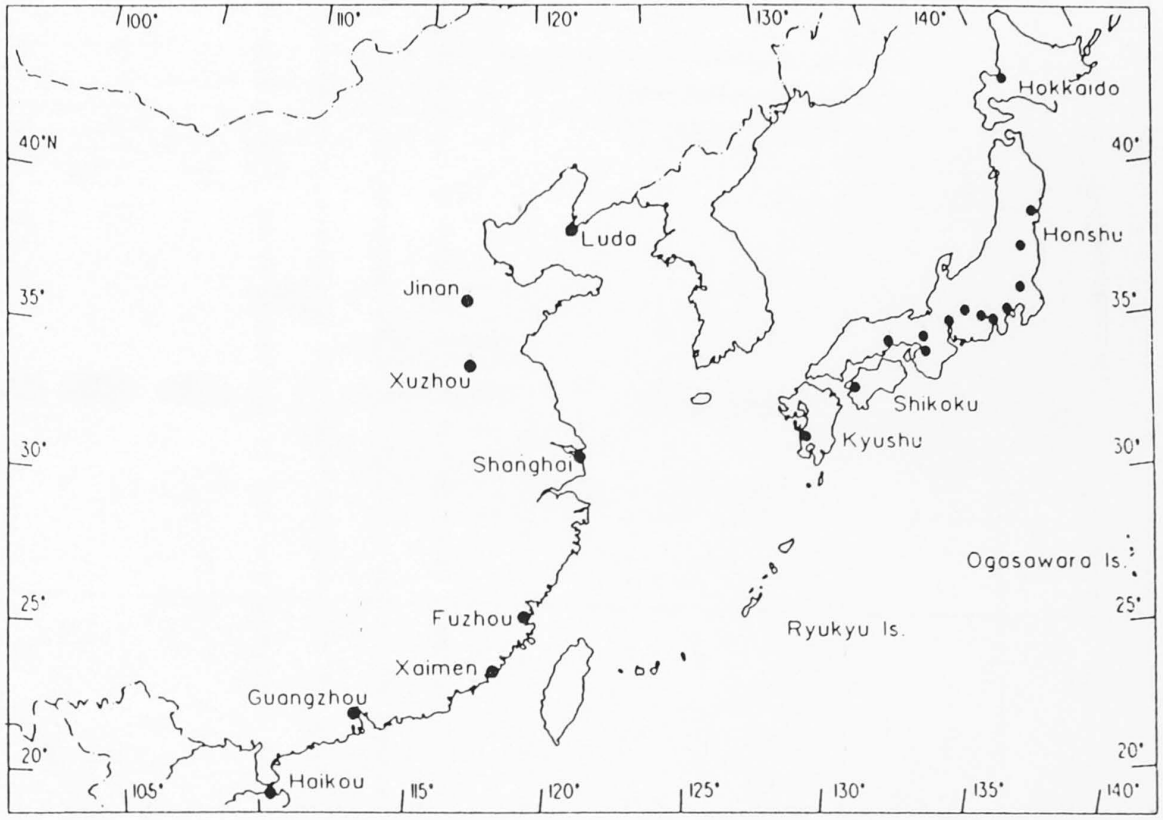

100°
110°
120°
130°
140°
40°N
40°
35°
30°
25°
20°
105°
115°
120°
125°
130°
135°
140°
Hokkaido
Honshu
Shikoku
Kyushu
Ogasawara Is.
Ryukyu Is.
Ludo
Jinan
Xuzhou
Shanghai
Fuzhou
Xaimen
Guangzhou
Haikou

Figure 2.2 Diagrammatic representation of the ADH staining regions on cellulose acetate sheets before and after incubation at 43°C for 15 seconds. The different shadings give an indication of the intensity of staining. The figure is taken from Wilks *et al.*, (1980).

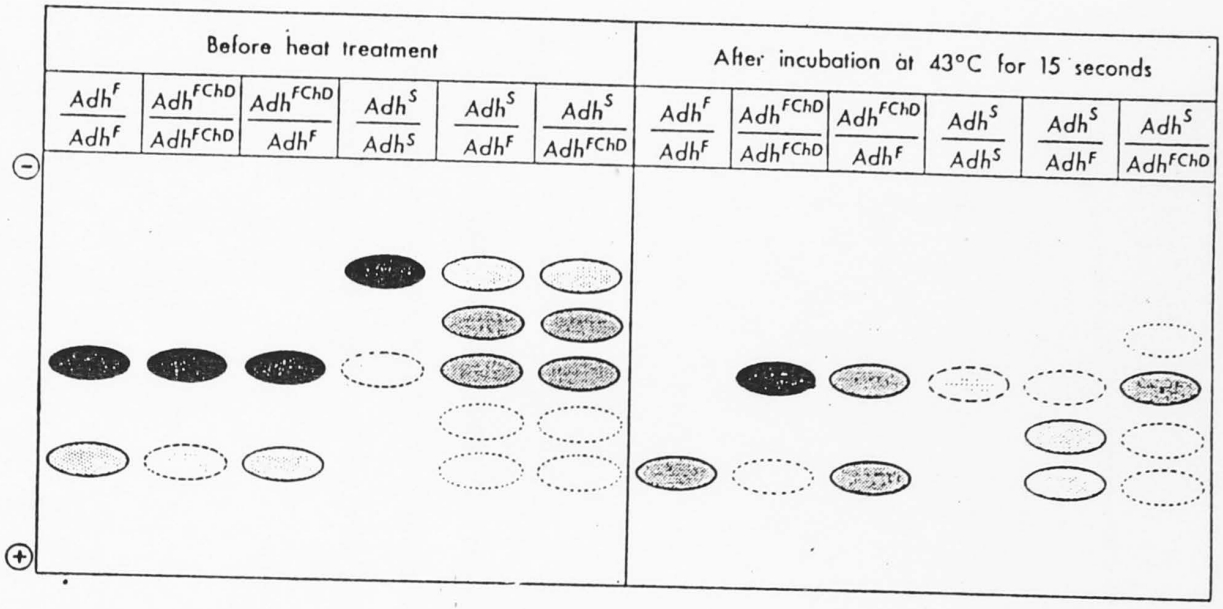
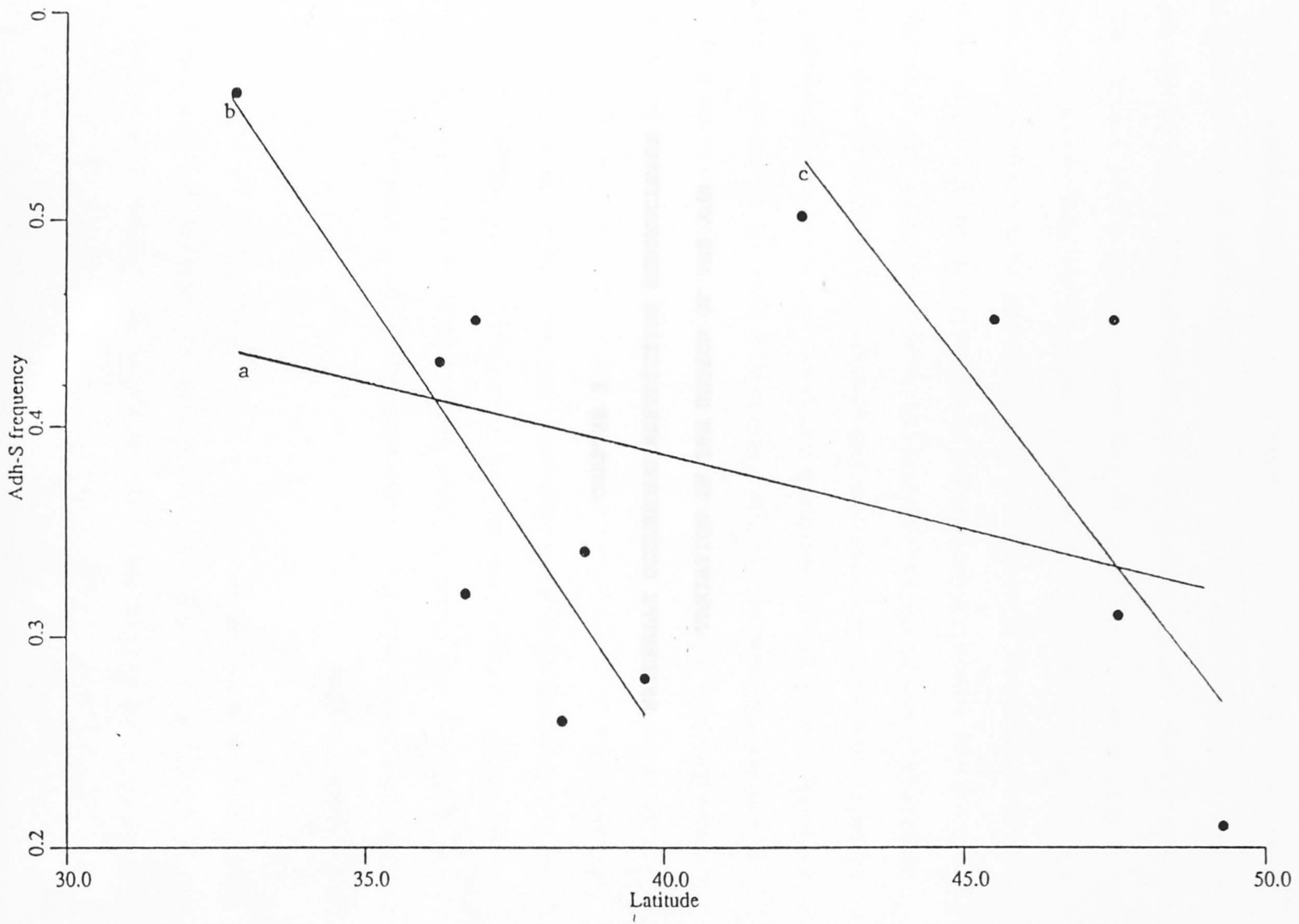

Before heat treatment
After incubation at 43°C for 15 seconds
Adh^F / Adh^F
Adh^FChD / Adh^FChD
Adh^FChD / Adh^F
Adh^S / Adh^S
Adh^S / Adh^F
Adh^S / Adh^FChD
⊖
⊕

Figure 2.3 *Adh*^{S} frequencies and their relations to latitude (linear regression) in the West Coast of U.S. (a).total data (b).data from California (c).data from Oregon, Washington ans British Columbia (data from Smith *et al.*, 1984).


Adh-S frequency
0.
0.5
0.4
0.3
0.2
b
a
c
30.0
35.0
40.0
45.0
50.0
Latitude

CHAPTER 3

NATURALLY OCCURRING RESTRICTION ENDONUCLEASE VARIATION IN THE REGION OF THE *Adh*

Chapter 3 Naturally occurring restriction endonuclease variation in the region of the *Adh* locus of *Drosophila melanogaster* in Australian and Chinese populations.

3.1 Introduction

The discovery of restriction fragment length polymorphisms (RFLP's) and the techniques of DNA sequencing have greatly enhanced studies of genetic variation in natural populations (Avise *et al.*,1979; Kreitman, 1983; Aquadro and Greenberg, 1983; Jeffreys *et al.*, 1984).

Studies of RFLP's allow a quick survey of variation in large DNA fragments from a number of samples. Such a survey can detect three categories of restriction map differences both in transcribed regions (exons) and in untranscribed regions (introns and untranscribed flanking sequences). These are the presence or absence of a particular restriction endonuclease recognition site, insertions and deletions of nucleotide bases. Restriction endonuclease data can be used to estimate sequence diversity in a defined DNA region among populations and species since the data directly reflects changes in DNA sequence.

RFLP's were first used in the genetic analysis of populations by Avise *et al.*,(1979). They examined mtDNA isolated from 23 samples of three species of the rodent *Peromyscus*. Their data demonstrated that there was detectable heterogeneity in mtDNA sequences within and between natural geographic populations of the species and this variation was used to estimate the relatedness between individuals and populations.

The molecular landscape of several loci in *Drosophila melanogaster* have also been studied recently. Brown (1983) constructed the

restriction map of a 25kb region around the 87A7 heat shock locus, which produces heat inducible protein (hsp70), in 29 third chromosome lines from a natural population "Raleigh" (North Carolina, U.S.A.), and found that a significant amount (67%) of restriction map variation was due to the insertion of large transposable elements, all the insertion events (four large (0.8-7kb) and two small (0.1-0.2kb) insertions) were found in a region less than 2kb. Only two restriction sites were found to be polymorphic. The estimated heterozygosity per nucleotide pair (θ) in this region was found to be 0.0024.

Langley and Aquadro (1987) investigated the restriction map variation of a 45kb region around the *white* locus, which is located on the X chromosome, in 38 lines from ten locations around the world. Their observations showed that the frequencies of individual large insertions were low, although 37% of the chromosomes had at least one insertion in this region. The estimated heterozygosity per nucleotide pair was 0.012, five times as high as that in the region of the 87A7 heat shock locus and twice as high as that in the region of *Adh* locus (see below). In contrast to the *Adh* region, there was little linkage disequilibrium among the polymorphic sites in the *white* region.

Schaeffer *et al.*(1988) investigated a 60kb region around the *Notch* locus in 37 X chromosomes from the same populations as used in the investigation of the *White* region. The *Notch* gene codes for a 10.5kb poly(A^+)RNA. Mutations in this gene are dominant lethal but in heterozygous females they disrupt the morphology of the wing and eye. Only four insertions and one deletion were observed in this region, and the amount of linkage disequilibrium was low. However the level of nucleotide substitution variation, θ=0.007, is similar to that in the region of the *Adh* locus.

The restriction endonuclease variation in the 15 kb region surrounding the *Amy* gene, which is duplicated in the genome and produces α-amylase, has been studied by Langley *et al.* (1988). In 85 lines from 15 populations around the world, large insertions were found in 12% of the lines, and their frequencies were low. The estimated heterozygosity per nucleotide pair θ=0.006. They found that allozyme variation was in linkage disequilibrium with three polymorphic restriction sites closely linked to the locus, and adult α-amylase activity was correlated with the allozymes and one polymorphic restriction site close to the transcriptional units.

Eanes *et al.*, (1989) investigated restriction map variation in the *G6pd* region in 126 lines from North American, European and African populations. They found clusters of insertions including some in the intron 1 region, however the nucleotide heterozygosity of this gene region is very low, θ=0.0035.

Aquade *et al.*, (1989) studied restriction map variation in a 20kb region of the *zeste* and *tko* genes. These two genes are closely linked (0.006 map units apart) on the X chromosome; *zeste* is a regulatory gene which affects expression of *bithorax, decapentaplegic and the white* genes, *tko* (technical knockout) is a behavioral mutation which causes "bang sensitivity" (Judd *et al.*, 1972). The levels of nucleotide heterozygosity and insertion/deletion variation in this region are similar to other gene regions on autosomal chromosomes. Little linkage disequilibrium between polymorphic sites was observed.

Previous work on the molecular landscape of the seven structural genes in *Drosophila melanogaster* is summarised in table 3.1.

Of particular interest to my research is the restriction variation in the region of the *Drosophila melanogaster Adh* gene, which has been cloned by Goldberg, D.A.(1980). Langley *et al.*,(1982) surveyed a 12kb

region of the *Adh* gene in 18 lines of *Drosophila melanogaster* from four American populations and five closely related species of *Drosophila: D.mauritiana, D.simulans, D.erecta, D.teissieri and D.yakuba*. They estimated the heterozygosity per nucleotide pair in *Drosophila melanogaster* to be 0.006. They also found that all of the detected restriction endonuclease differences were outside the *Adh* coding region. The insertions/deletions that they detected were each restricted to local populations, whereas the restriction site polymorphisms were more widely distributed. They argued that the insertions/deletions may be mildly deleterious.

Birley (1984) surveyed the same region of the *Adh* gene in 16 highly inbred lines extracted from the "Texas" cage population. He found that the heterozygosity per nucleotide pair was 0.004 in these lines and two major classes of haplotype were strongly associated with the capacity of the lines to change ADH activity in response to environmental factors. Cross and Birley (1986) studied restriction variation in the *Adh* region in 59 isochromosomal lines extracted from the population "Groningen" (Holland) and "Chateau Tahbilk" (Australia). The heterozygosities per nucleotide pair were 0.005 and 0.007 for "Chateau Tahbilk" and "Groningen" respectively. A 1kb region, approximately 4kb from the 5′ transcriptional unit, was identified as an insertion/deletion cluster region.

Aquadro *et al.*,(1986) used six and four base cutting restriction enzymes to analyze 48 lines representing four eastern United States populations of *D.melanogaster*. Their analysis revealed extensive DNA sequence variation and strong nonrandom associations among some of the variants. Recently Simmons *et al.*,(1989) used four-cutter analysis to investigate the restriction map variation of a 2.7kb region containing the *Adh* structural locus in 270 lines from three populations along the

east coast of the United States. The data indicated there were no significant differences between the three populations in restriction site polymorphisms. They found that the main haplotype difference between populations was due to the differing frequencies of Adh^F and Adh^S.

Apart from the Simmons *et al.* (1989) study there has been no systematic geographic survey of restriction endonuclease variation in the region of the *Adh* gene of *Drosophila melanogaster*. Hence it has not been possible to investigate in detail any relationships between restriction site variation and environmental gradients. The frequency of the Adh^F gene of *Drosophila melanogaster* exhibits a latitudinal cline in different continents (see Chapter 2). The cline might be maintained by selection on the locus, but it could also be due to selection acting on some factor tightly linked to the locus, such as an insertion or deletion in the region of *Adh* gene. An investigation of restriction endonuclease variation in the *Adh* gene region of *Drosophila melanogaster* in populations from two continents (Australia and mainland China) was carried out to test the above possibility.

The work described in this chapter is concerned with three questions:

(1) Does restriction endonuclease variation parallel the latitudinal cline in Adh^F frequency?

(2) Is there heterogeneity in restriction endonuclease variation between populations, and is it similar in populations at the same latitude on different continents?

(3) Is there any indication of the extent of migration between populations, in that geographically related populations have similar insertions or deletions?

In order to answer these questions 90 second chromosomes from six Chinese populations and 104 second chromosomes from seven Australian populations (see Figure 3.1.1 and 3.1.2) were analysed for restriction endonuclease variation in a 12kb region encompassing the *Adh* structural gene locus.

3.2 Materials and Methods

3.2.1 Extraction of single chromosome lines

A large number of flies were collected from seven Australian and six Chinese natural populations in October and May, 1986 and 1987. The Chinese samples were collected by H. Chen and air freighted to Canberra as described in chapter 2. From each population single adult female flies were isolated in vials and cultured on high protein food (agar 10g, corn syrup 30ml, high protein powder 10g, malt 40g, sucrose 15g and yeast 35g in 1 liter of water with 4.5ml of propionic acid) and grown at 25°C. After the progeny emerged, one adult male was picked and crossed with a virgin female of a second-third chromosome translocation stock *T(2;3) ap^{Xa}*, which contains the second chromosome balancer *Cy0*, and the third chromosome balancer, *TM6* (Lindsley and Grell, 1968). From this single pair mating (Figure 3.2.1) one curly wing male was back crossed with several translocation stock virgin females. From the F_2 progeny *Cy* phenotype males and virgin females were chosen and put into vials to breed. From the progeny wild type males and virgin females were mated together to make a line which was homozygous for a single second chromosomes. Any homozygous lethal chromosomes were maintained heterozygous with *Cy*. 15 single chromosome lines were extracted from each population.

3.2.2 Genomic DNA extraction

Two methods were used in the present study to extract genomic DNA.

(1) CsCl gradient centrifugation (Miklos, 1984)

1-1.5g adults flies were frozen in liquid nitrogen and homogenised in buffer (10mMTris, pH8.0, 20mM EDTA), and then lysed with the detergent Sarkosyl NL-30 (final concentration 0.3%). Lysis was completed by slowly inverting the glass tube. The lysate was added directly to Ti 50 centrifuge tubes containing solid caesium chloride (final concentration 1g/ml). Ethidium bromide was added to the mixture to a final concentration of 0.6mg/ml. After the caesium chloride had dissolved, the tube was spun in a Beckman L5-65 centrifuge at 40,000rpm at 10°C for 40-45 hours. Following centrifugation the main genomic DNA band was visualised under ultra-violet (U.V.) light (366nm), and collected by side puncture with a 19 gauge needle. The ethidium bromide was removed by 5-6 extractions with isopropanol. The DNA was dialysed against T.E. buffer (10mM Tris-HCl, pH8.0, 1mM EDTA) overnight. The concentration of DNA solution was determined by reading the absorption of U.V. at 260nm. The concentration was usually about 100µg/ml. The DNA was also checked by electrophoresis in 1% agarose to make sure it was not degraded. The DNA was stored in an sterile Eppendorf tube at -20°C.

(2) Rapid DNA extraction (Cooke, P.H. personal communication)

50 adults flies were homogenized in an Eppendorf tube in 50µl cold buffer (100mM Tris, PH9.0, 100mM EDTA). 500µl of 2% SDS was added to the homogenate and mixed well. The tube was incubated in a 65°C water bath for 1 hour. After the tube had cooled to room temperature, 120µl of 8M potassium acetate was added, mixed well and left on ice for 30 minutes. The tube was then spun in an Eppendorf centrifuge at 4°C for 10 minutes. The supernatant was transferred to a clean Eppendorf tube and 500µl of isopropanol was added, mixed well by inversion then spun

at room temperature for 10 minutes. A pink pellet was seen in the bottom of the tube. The pellet was washed twice with cold 70% ethanol, dried in vacuum and resuspended in 50µl T.E. buffer with 20µg/ml boiled RNAse. The concentration of the DNA was usually about 100-150µg/ml.

3.2.3 Plasmid probe

The recombinant plasmid sAF2 used was originally obtained from Dr. D.A. Goldberg. Plasmid sAF2 contains a 11.8kb *Sac*I fragment, which was derived from the *Adh* region of a homozygous Adh^F strain, which had been cloned into the bacteriophage Charon 10 and later subcloned into a pBR322-derived *Sac*I vector(pSV2) to give the sAF2 clone. pSV2 had been constructed by placing a central EcoRI fragment from Charon 9 into pBR322 and subsequently removing the internal *Sac*I fragments(Goldberg, 1980). I checked the restriction map of the *Sac*I fragment in sAF2 by measuring the sizes of fragments from digestions with six restriction endonucleases: *Hin*dIII, *Eco*RI, *Sac*I, *Hpa*I, *Sal*I and *Bam*HI. The sizes of these fragments are shown in Figure 3.2.2 and are consistent with Goldberg's restriction map of sAF2 which is shown in Figure 3.2.3.

3.2.4 Isolation of plasmid DNA (modified after Maniatis *et al.*, 1982)

Escherichia coli carrying recombinant plasmids were seeded into L broth media (bactotryptone 10g, yeast extract 5g, NaCl 0.5g in 1 liter distilled water) in the presence of 0.2g/L $MgCl_2$ and ampicillin (50µg/ml), and incubated in a shaker at 37°C overnight. 7.5ml of the culture was diluted 1 : 100 with the same media and incubated further for 3-4 hours. The OD_{600} reading of the culture was checked regularly until it reached 1.00, when 100µg/ml chloramphenicol was added. The

culture was incubated overnight at 37°C. Plasmid-containing bacteria were recovered by centrifugation in polypropylene bottles at 7000rpm for 5 minutes. Following this the supernatant was discarded and the pellet was resuspended in 40 ml of GTE solution(50mM Glucose, 25mM Tris, pH8.0, 10 mM EDTA) with 2mg/ml lysozyme and put on ice for 30 minutes. 75ml of fresh 0.2M NaOH/1% SDS was added and mixed well, before the addition of 40ml of cold 3M potassium acetate/5M acetic acid. This was mixed gently until no dark gelatinous material remained, and left on ice for at least 15 minutes, followed by centrifugation at 9000rpm for 20 minutes in a GSA rotor. On completion the supernatant was poured through a sterile macrocloth into a fresh bottle containing 0.6 volume (90 ml) of isopropanol, and mixed well to precipitate the DNA. The bottle was left at -20°C for 30 minutes, and then spun at 7000rpm for 10 minutes in a GSA rotor, after which the supernatant was discarded and the pellet dried in vacuum for 30 minutes. The pellet was dissolved in 7.4 ml of T.E. buffer, pH 8.0. 8.4 ml of the DNA solution was added to 10g CsCl with 1.6ml 10mg/ml ethidium bromide, mixed well, spun in a Ti50 tube at 40000rpm, at 10°C for 40 hours. The plasmid DNA band was recovered using a needle and a syringe and the ethidium bromide was extracted using 1-butanol (3-4 times). The DNA was precipitated by the addition of two volumes of 100% cold ethanol and washed with 80% ethanol,dried in vacuum then resuspended in T.E. buffer and stored in -20°C.

3.2.5 DNA sample digestion and electrophoresis

DNA extracted from single chromosome lines was digested by restriction endonucleases under the conditions recommended by the manufacturer (Amersham). Eight restriction endonucleases, (*Bam*HI, *Eco*RI, *Hind*III, *Hpa*I, *Pst*I, *Sal*I, *Xba*I and *Xho*I), all specific for

different hexanucleotide sequences, were used. These restriction endonucleases had previously been shown by other authors to be polymorphic in the region of the *Adh* gene in *Drosophila melanogaster* populations. Double and triple endonuclease digestions were made to locate any variation found. About 0.5-1µg of genomic DNA was added to the digestion mixture, which was then incubated in a 37°C water bath for 2 hours. The reaction was stopped by adding sample dye (30% sucrose, 0.09% bromophenol blue, 50mM EDTA). Electrophoresis was carried out in a 1% agarose gel in a horizontal gel apparatus. The electrophoresis buffer contained 0.04M Tris-HCl, pH7.8, 5mM sodium acetate, 1mM EDTA. The gel was run for 14-16 hours at 40 mA (about 35V) in the presence of ethidium bromide (2µg/ml) and the DNA bands were visualised under U.V. light (254nm). Lambda DNA (c1857, Boehringer) digested with *Hin*dIII was used to provide size markers.

3.2.6 Hybridisation of Southern blots

Southern blotting (Southern, 1975) was used to detect specific DNA sequences and construct the restriction maps of the *Adh* region. After electrophoresis the gel was trimmed and transferred to a denaturing solution, Blot I (0.8M NaCl, 0.4M NaOH), for two washes of 45 minutes each at room temperature, followed by neutralisation by two washes of 45 minutes each in a Blot II solution (1.5M NaCl, 0.5M Tris-HCl, pH7.4). Transfer of DNA from the gel to a nitrocellulose membrane (Schleicher and Schull BA85) was completed by blotting in 20X SSC (2.7M NaCl, 0.27M sodium citrate) for about 20 hours. The nitrocellulose membrane was washed in 2X SSC for 5 minutes then baked for 3-4 hours at 80°C under vacuum.

The sAF2 plasmid was labelled with α^{32}PdCTP, 3000Ci/mM (Amersham International) by nick-translation. 3µl of α^{32}PdCTP(30uCi) was added

to the following mixture: 3µl of 20mM dATP, 20mM dGTP, and 20mM dTTP, 10 units of DNA polymerase I, 2µl(0.1µg/ml) of DNAase I, 1-1.5µg plasmid DNA, in a total volume of 50µl in N.T. buffer(500µl 1MTris-HCl, pH7.2, 100µl 1M $MgSO_4$, 1µl 1M DTT, 250µl 2mg/ml BSA and 150µl H_2O make 1ml of 10X N.T.buffer). The mixture was incubated at 14-15°C in a circulating water bath for 2 hours and then put through a 3 ml Sephadex-G50 (Pharmacia) column to remove unincorporated nucleotides. Radiolabelled DNA was eluted from the column by centrifugation (2000rpm for 5 minutes) and a Geiger counter reading on a 100µl sample was used to check incorporation. The hybridisation probe was stored at -20°C until required.

Prehybridisation of the nitrocellulose membrane was carried out in a sealed plastic bag for 1 hour at 65°C in 3X SSC containing 10X Denhardts solution (Denhardts, (1966): 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone 40), 0.1% SDS, 0.1% tetra sodium pyrophosphate, with 30µg/ml of heat denatured herring sperm DNA. Hybridisation was carried out at 65°C for 14-16 hours in the same bag after the addition of the radiolabelled probe, which had been denatured in a 100°C water bath for 5 minutes. After hybridization the filter was washed three times for 45 minutes each at 65°C in 2X SSC with 0.1% SDS and 0.1% tetra sodium pyrophosphate. The filter was air dried at room temperature for two hours, and then exposed to Kodak X-ray film(XRP-1) using intensifying screens at -70°C for 48-72 hours. The fragments which hybridized to the labelled probe were visualised after the film was developed using Kodak developer type 2 and Ilford quick fixer. The sizes of the visualised bands were determined graphically from a semi-logarithmical plot of standard Lambda DNA fragments (digested by *Hin*dIII) sized in kb units versus relative mobility in cm. The fragment size data were used to construct restriction maps.

3.3 Results

The restriction maps of the probed region containing the *Adh* gene in seven Australian populations and six Chinese populations are illustrated in Figure 3.3.1.a,b; and listed in Table 3.3.1 and Table 3.3.2. These data are derived from 90 chromosomes from Chinese populations and 104 chromosomes from Australian populations.

To facilitate comparison with previous work on the *Adh* gene region, the *BamHI* site in the second exon of the *Adh* transcriptional unit has been given the co-ordinate of 0.0.

Overall in the Chinese populations, 12 polymorphic restriction sites: *Eco*RI(-8.6), *Xba*I(-7.4), *Bam*HI(-7.2), *Hpa*I(-6.9), *Xba*I(-5.3), *Hin*dIII(-3.7), *Hin*dIII(-3.0), *Pst*I(1.1), *Xho*I(1.2), *Pst*I(1.3), *Hin*dIII(2.5), and *Eco*RI(9.0), and six types of insertion or deletion: insertion 0.35kb, 1.5kb, 0.4kb, 0.48kb, 0.7kb, deletion 0.2kb, were present. In the Australian populations 13 polymorphic restriction sites:*Xba*I(-8.0), *Xba*I(-7.4), *Bam*HI(-7.2), *Hpa*I(-6.9), *Xba*I(-5.3), *Hin*dIII(-3.7), *Hin*dIII(-3.0), *Pst*I(1.1), *Xho*I(1.2), *Xba*I(1.4), *Bam*HI(3.9) and *Eco*RI(9.0), and eight types of insertion or deletion: insertion 0.28kb, 5.0kb, 0.4kb, 0.7kb, 1.0kb, 3.0kb, 4.5kb, deletion 0.2kb were present. Ten of the polymorphic restriction sites were shared by populations on both continents, but only three of the 11 different insertions or deletions were shared. Deletion 0.2kb and insertion o.4kb occur in nearly all the populations surveyed. Each of the insertions larger than 1kb was unique to particular populations, although they were sometimes found in more than one single second chromosome line. Restriction site variation in natural populations is more universal than insertions, especially large insertions.

In the survey one chromosome (T40) exhibited a very different restriction map pattern in the *Adh* region (Figure 3.3.2). The size of the sequence hybridising to the probe sAF2 was 16kb. The restriction map of this chromosome showed that parts of the *Adh* coding region, from *Hpa*I(-0.9) site to *Bam*HI(0.0) site, and in the 5′ end region, from *Bam*HI(-7.2) site to *Pst*I(-5.4) site were duplicated.

Estimates of nucleotide-substitution variation and haplotype diversity in the Chinese and the Australian populations are shown in table 3.3.4. The estimate of the proportion of polymorphic nucleotides (the probability that two or more nucleotide types appears in the sample) was calculated using equation 13 from Ewens *et al.*(1981) and from equation 7 from Hudson(1982): $P=k/(2m-k)j$,

where m = total number of cleavage sites of the restriction endonuclease,

k = number of polymorphic cleavage sites,

j = the length of the recognition sequence of the restriction enzyme.

The variance of P was calculated using equation 11 in Hudson (1982): $Var(P)=P^2/k$.

The estimated heterozygosity per nucleotide pair, θ (the probability that when two homologous DNA sequences are compared they will show different nucleotide types at a given site) was calculated using equation 11 in Ewens *et al.*(1981): $\theta=k/(12mlnN)$, where N is the number of samples. The variance of θ was calculated using equation 19 in Hudson(1982): $Var(\theta)=\theta^2/k$.

The haplotype diversity was estimated using the equation in Nei and Tajima (1981): $h=n(1-\Sigma X_i^2)/(n-1)$, here n is the number of samples, and X_i is the frequency of ith haplotype in the population. The variance of h is: $Var(h)=2M/(1+M)^2(2+M)(3+M)$, where $M=h/(1-h)$.

These estimates show that, overall, there is a higher level of nucleotide-substitution variation in the Australian populations (on average one in 47 nucleotides is polymorphic) than in the Chinese populations (one in 70 nucleotides is polymorphic). Between any two randomly chosen chromosomes one in 127 nucleotide sites will differ in the Australian populations and one in 189 nucleotide sites in the Chinese populations. The averages of the estimates of haplotype diversity in the Australian and the Chinese populations are shown in table 3.3.5. The overall heterozygosities for the two sets of populations are quite similar when all types of variation are considered (0.96 vs 0.97).

The level of haplotype diversity in Adh^S bearing chromosomes showed a slightly higher heterozygosity than Adh^F bearing chromosomes in populations from both countries (0.80 vs 0.78 in China; 0.97 vs 0.90 in Australia); the Adh^F heat-resistant allele bearing chromosomes had the lowest estimated heterozygosity (0.64).

The data summarised in table 3.3.4 provide no evidence for any consistent relationship between latitude and the level of nucleotide-substitution variation or with the level of haplotype diversity.

None of the restriction site polymorphisms, nor any of the insertions, showed significant associations with latitude. However the 0.2kb deletion, which was present in all Australian and Chinese populations, except the Tamar population, increased in frequency towards the equator in the Chinese populations but not in the Australian populations. Table 3.3.3 and Figure 3.3.2 show the regressions of the frequencies of deletion 0.2kb on latitude.

Nonrandom associations of variants in the *Adh* region are listed in table 3.3.6. Tests of linkage disequilibria were carried out by Fisher's exact test for a 2x2 contingency table. Any variant with a

frequency less than 15% or more than 85% was excluded from this analysis. The most common significant nonrandom association is between the *Bam*HI(-7.2) site and Adh^S, which occurred in eight out of the ten populations in which the frequency of BamHI(-7.2) is greater than 15%. Another common kind of association is between the presence of insertions/deletions and some restriction sites, such as the nonrandom association between *Hin*dIII(-3.0) and D(0.2), which occurred in three populations.

3.4 Discussion

Restriction fragments of less than 50bp will not be detected by the agarose gel electrophoresis techniques I have used. This resolution is similar to that obtained by Langley *et al.* (1982), Birley (1984) and Cross and Birley (1986), but less than that obtained by Aquadro *et al.* (1986), who used different concentrations of agarose gel and found insertions/deletions less than 50bp. I have used 0.5% and 2% agarose gels, but still cannot obtain a resolution less than 50bp.

The restriction endonucleases I chose to use in my study had previously been shown to be polymorphic in other populations by other workers. Thus it was not surprising that the most common restriction endonuclease site variants found in the Chinese and the Australian populations were similar to those reported previously in other populations: *Bam*HI(-7.1), *Hin*dIII(-3.0), *Hin*dIII(-2.7) and *Xho*I(1.2) were found to be polymorphic by Langley *et al.*,(1982); *Pst*I(-6.2), *Hin*dIII(-3.0), *Xho*I(1.2) and *Bam*HI(1.5) by Birley (1984); *Bam*HI(-7.0), *Hin*dIII(-2.7), *Pst*I(1.0), *Xho*I(1.5), *Pst*I(2.0), *Eco*RI(3.7) and I(9.0) by Cross and Birley, (1986); *Bam*HI(-7.1), *Eco*RI(-4.5), *Hin*dIII(-2.7) *Hin*dIII(0.8) and *Xho*I(1.9) by Aquadro *et al.* (1986). The locations of

the polymorphic sites may differ slightly between authors because of errors in measuring fragment sizes.

The most common insertion and deletion, I(0.4) and D(0.2), I found in the Australian and Chinese populations, were also reported in Aquadro *et al.* (1986), and insertions/deletions of similar sizes and locations were reported in Birley (1984) and Langley *et al.*,(1982). The other insertions in the Australian and Chinese populations vary in size from 0.28 to 5.0kb and occur in low frequencies usually found only once in the samples from any population investigated. This was also found in the previous investigations. The insertions, particularly the large insertions, seem quite local. They may have selective disadvantages which prevent them from spreading widely. Interestingly, in all reports mentioned above there is a cluster of insertions, which vary in size, occurring in the 0.23kb region about 3.5 kb from the 5'end of transcriped unit, e.g. in the region between the *Eco*RI and the *Sal*I site of the 3' flanking sequence (see Figure 3.3.1 a,b,). The distribution of insertions which are larger than 50bp in the *Adh* gene region is shown in Figure 3.4.1. Collating my data with previous data, 70% of the insertions in 335 lines occurred in the "hot spot" (Figure 3.4.2). It may be that sequences in this "hot spot" are recognised by some particular mobile elements. Insertions in the "hot spot" usually have no effects on ADH activity (see chapter 6), and seem to be neutral or slightly deleterious.

No restriction site polymorphisms were found in the *Adh* coding region, except for the nucleotide substitution responsible for the electrophoretic phenotypes Adh^F *and* Adh^S. One insertion, I(5.0kb), was found in intron I in one chromosome. In the regions from -5.0 to -4.0kb and from the 3' end of the Adh coding region to +1.1kb, little variation was present. These two regions have been tentatively

identified as functionally important regions. Cohn *et al*(1988) and Kreitman and Aquade (1986) suggested that the characteristics of the two regions implied the presence of two putative loci. In their population survey using sequence comparisons and four-cutter restriction endonuclease analyses, Kreitman and Aquade (1986) found an excessively high level of silent mutation polymorphism in the *Adh* coding region, equivalent to that found in the 5′ flanking region. They explained this observation as being due to the recent loss of constraints within the locus or to the selective maintenance of the ADH protein polymorphism. An interspecific sequence comparison between the sibling species *D.melanogaster* and *D.simulans* showed a twofold higher level of divergence in the 5′ flanking sequence compared to the structural locus. This suggested that in the process of speciation the functionally important region is more conserved. In contrast to previous data on other gene regions, a study of restriction map variation in *G6pd* revealed a cluster of big insertions in the large intron 1 of this gene. Eanes *et al.* (1989) found that two big insertions, 4.2kb (in intron 1) and 12.5kb (in the 3′ flanking sequence) in the region of *G6pd* reached high frequencies and were found across a considerable geographic distance: the frequency of a 4.2kb insertion is 32% in European populations and common in Japanese populations (personal communication between Eanes and Hori, S.), whilst the frequency of a 12.5kb insertion is 11% in the North American populations. The nucleotide heterozygosity of this gene is very low (θ = 0.0035) compared with other gene regions which has been studied.

The levels of nucleotide-substitution variation and haplotype diversity in the seven Australian and six Chinese populations vary as shown in table 3.3.3. Variation of p ranges from 0.12 to 0.24, h from

0.76 to 1.00 and θ from 0.0036 to 0.009. The values of p, h and θ in the populations of the two countries are similar to the data previously published (table 3.4.1): θ=0.006 in Langley *et al.* (1982); p=0.012, h=0.86 and θ=0.004 in Birley (1984); p=0.017, h=0.94 and θ=0.005 in "Chateau Tahbilk", P=0.025, h=0.665 and θ=0.007 in "Groningen" in Cross and Birley (1986) and θ=0.006 and h=0.97 in Aquadro *et al.* (1986). However a comparison of nucleotide-substitution variation and haplotype diversity between Australian and Chinese populations showed that there was higher p, h, and θ in Australian populations (averages of seven Australian populations: p=0.021, h=0.96, θ=0.008) than in Chinese populations (averages of six Chinese populations: p=0.014, h=0.88, θ=0.005). The estimates of nucleotide-substitution variation (p and θ) are 50% higher in the Australian populations. This difference might be related to the historical origins of the populations. David and Capy (1988) have suggested that world populations may be divided into the three categories:"ancestral"-populations in tropical Africa where it is believed *Drosophila melanogaster* originated,"ancient"-populations derived from the initial colonization of Eurasia without the agency of man and "new"-populations introduced by modern man. The Chinese populations belong to "ancient" populations. However Australia was colonized mainly from a mixture of African and European populations following European settlement, no more than two centuries ago. Thus the "new" Australian populations might be expected to be more genetically heterogeneous than the "older" Chinese populations. However, the overall comparison of heterozygosity (h) between populations in the two countries is not significantly different (h=0.97 in the Australian population and h=0.96 in the Chinese population).

Overall, in the Australian and Chinese populations the level of heterozygosity is higher for the haplotypes with Adh^S than for those with Adh^F. Adh^S is arguably the ancestral allele (Kreitman, 1983; Ashburner *et al.* 1984; Bodmer and Ashburner, 1984; Cohn *et al.* 1984 and Stephens and Nei, 1985); Adh^F was derived from Adh^S (threonine replaced lysine in position 192) and thus more variation could have accumulated in the region of the chromosomes bearing the Adh^S allele. Ashburner *et al.* (1984) provided strong evidence supporting the hypothesis that Adh^S is the ancestral allele. They compared the nucleotide sequences of the *Adh* gene between *D. melanogaster* and three of its sibling species, *D. simulans, D. mauritiana and D. orena*, and found that the *Adh* genes of all three species have the "Adh^S" lysine in position 192 rather than the "Adh^F" threonine. In a comparison of 17 sites (from -3 to 1557) which represent reasonably consistent changes in the "consensus" sequences (in both coding and noncoding regions) distinguishing *Adh* slow from fast alleles of *Drosophila melanogaster* (Kreitman, 1983), Ashburner *et al.* (1984) found that at most of these sites (ie.14/17) the sequence of the *Drosophila simulans Adh* gene resembles Adh^S rather than Adh^F. Ashburner *et al.* estimated Adh^F may have arisen 1-3 million years ago. Kreitman(1983) compared the sequences of eleven cloned *Adh* genes, five Adh^F and six Adh^S genes, and found the Adh^F alleles to be more homogeneous than the Adh^S alleles. In the present study the Adh^F heat-resistant alleles have the lowest heterozygosity suggesting that this allele is of more recent origin. This observation is consistent with the amino acid sequence data (Chambers *et al.*, 1981) which showed that, compared to ADH-F, the enzyme produced by the heat resistant allele, Adh^{FChD}, has an additional replacement of serine for proline at residue 214. It is also consistent with the DNA sequence data of Collet (1988). He

determined the nucleotide sequence of Adh^{FChD} for 3kb spanning the *Adh* gene and observed the Adh^{FChD} sequence has the base substitution of T for C at position 1555 which is responsible for the amino acid replacement at residue 214. He also observed that Adh^{FChD} has the base C at position 1490 which is the same as Adh^{F}, but distinguishes Adh^{F} from Adh^{S}. In a comparison of the sequence of the region -63bp to +2000bp with the consensus sequence of six Slow and five Fast haplotypes sequenced by Kreitman (1983), he found that the Adh^{FChD} sequence differed from the "consensus" sequences of Adh^{F} and Adh^{S} by 5 and 19 nucleotides respectively. From these data he estimated the time of divergence of the Adh^{FChD} sequence from Adh^{F} is to be approximately 260,000-470,000 years ago.

Aquadro *et al.* (1986) using the linkage disequilibrium data between restriction site *Bam*HI(-7.2) and the *Adh* (F/S) allozyme substitution site, estimated Adh^{F} arose even more recently, about 2000 years ago. Complete linkage disequilibrium between the two sites was found in 48 lines from four eastern United States populations, and also in 18 lines from several American populations (Langley *et al.*, 1982). In the present study the most consistent nonrandom association is also between the same two variants. It is found in eight out of the ten populations in which the frequency of the *Bam*HI(-7.2) site was higher than 0.15. The linkage is not as tight as in the American populations as the level of significance varies, and in some populations (Coffs Harbour and Cygnet) the association is not significant.

Table 3.1 Summary of previous work on restriction endonuclease variation in the region of structural genes of *Drosophila melanogaster* in natural populations.

locus	region (kb)	populations					
		Europe	N.America	Australia	S.America	Africa	Asia
Adh	12	Groningen(34)[c]	Rhode Island(10)[a]+(23)[d] kansas(4)[a] +(11)[d] Minnesota(2)[a] North Carolina(2)[a]+(6)[d] Texas(16)[b] Wisconcin(6)[d]	Tahbilk(25)[c]			
Amy	15	France(7)[e]	Kansas(13)[e] Wisconsin(9)[e] North Carolina(12)[e] Rhode Island(23)[e] Ottawa(2)	Fairfield(3)[e]	Argentina(3)[e] Belieze(2)[e]	Benin(2)[e] Central Africa(1)[e]	Japan(1)[e] Taiwan(2)[e] Hawaii(1)[e] Vietnam(4)[e]
Notch	60	France(7)[f]	North Carolina(5)[f] Wisconsin(4)[f] Ottawa(1)[f]	Fairfield(2)[f]	Angentina(3)[f] Belieze(2)[f]	Benin(2)[f] Central Africa(1)[f]	Hawaii(1)[f] Taiwan(2)[f] Vietnam(3)[f]
87A Heat Shock	13		North Carolina(29)[g]				
White	45	France(7)[h]	North Carolina(5)[h] Wisconsin(4)[h] Ottawa(2)[h]	Fairfield(3)[h]	Argintina(3)[h] Belieze(2)[h]	Benin(2)[h] Central African(1)[f]	Hawaii(1)[h] Taiwan(2)[g] Vietnam(4)[h]

continue

G6pd	13	France(15)[i]	New York(42)[i]	Botswana(27)[i]
		west Germany(10)[i]	California(32)[i]	
zeste-	20		California(20)[j]	Japan(17)[j]
tko			Texas(27)[j]	

a:Langley, et al.,1982. b:Birley, 1984. c:Cross and Birley, 1986. d:Aquadro, et al.,1986. e:Langley, et al.,1988. f:Schaeffer, et al.,1988. g:Brown, 1983. h:Langley and Aquadro, 1987. i:Eanes, et al.,1989. j:Aquade, et al., 1989.

Table 3.3.1 Restriction endonuclease variation in the *Adh* gene region of the Chinese natural populations of *Drosophila melanogaster*. I denotes an insertion, D a deletion, E-*Eco*RI, X-*Xba*I, B-*Bam*HI, P-*Pst*I, X-*Xho*I, h-*Hpa*I. The size of the insertion/deletion and the position of the restriction sites are indicated in parentheses. A denotes the *Adh* allotype (F-fast, S-slow, F^r-heat-resistant allele). The populations are: H-Haikou, G-Guangzhou, F-Fuzhou, S-shanghai J-Jinan, D-Dalian.

	E (-8.6)	X (-7.4)	B (-7.2)	I (0.35)	h (-6.9)	X (-5.3)	H (-3.7)	H (-3.0)	I (1.5)	D (o.2)	A	P (1.1)	X (1.2)	P (1.3)	I (0.4)	I (0.48)	I (0.7)	H (2.5)	E (9.0)
H 1	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	+
H 2	-	-	-	+	-	+	-	-	-	+	F^r	-	+	-	+	-	-	+	-
H 3	-	-	-	-	-	-	-	-	-	+	F^r	-	+	-	-	-	-	-	-
H 4	-	-	-	-	-	-	-	-	+	+	F	-	+	+	-	-	-	-	-
H 5	-	-	-	-	-	-	-	-	-	-	F	-	+	-	+	-	-	-	-
H 6	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
H 7	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
H 8	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	+
H 9	-	-	-	-	-	-	-	-	-	+	S	-	+	-	-	-	-	-	-
H 10	-	-	-	-	-	-	-	+	-	+	S	-	+	+	+	-	-	-	+
H 11	-	-	-	-	-	-	-	-	-	-	S	-	+	+	+	-	-	-	-
H 12	-	-	-	-	-	-	-	+	-	+	S	-	+	+	+	-	-	-	+
H 13	-	-	-	-	+	-	-	+	-	+	S	-	+	+	+	-	-	-	+
H 14	-	-	-	-	-	-	-	-	-	+	S	-	+	-	-	-	-	-	+
H 15	-	-	-	-	-	-	-	+	-	+	S	-	+	-	-	-	-	-	+
G 1	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	+
G 2	-	-	-	-	-	-	-	-	-	+	F^r	-	+	-	-	-	-	-	-
G 3	-	-	-	-	-	-	-	+	-	+	F	-	+	-	-	-	-	-	+
G 4	-	-	-	-	-	-	-	+	-	+	F	-	+	-	-	-	-	-	-
G 5	-	-	-	-	-	-	-	+	-	+	F	-	+	-	-	-	-	-	-
G 6	-	-	-	-	-	-	-	-	-	+	F^r	-	+	-	-	-	-	-	-
G 7	-	-	+	-	-	-	-	+	-	+	F	-	+	-	-	-	-	-	-
G 8	-	-	-	-	-	-	-	+	-	+	F	-	+	-	-	-	-	-	-
G 9	-	-	+	-	-	-	-	-	-	+	S	-	+	-	-	-	-	-	-
G 10	-	-	+	-	-	-	-	-	-	+	S	-	+	-	-	-	-	-	-
G 11	-	-	-	-	-	-	-	+	-	+	S	-	+	+	+	-	-	-	-
G 12	-	-	+	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-
G 13	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
G 14	-	-	-	-	-	-	-	-	-	+	S	-	+	-	-	-	-	-	+
G 15	-	-	-	-	-	-	-	-	-	+	S	-	+	-	-	-	-	-	-

	E (-8.6)	X (-7.4)	B (-7.2)	I (0.35)	h (-6.9)	X (-5.3)	H (-3.7)	H (-3.0)	I (1.5)	D (o.2)	A	P (1.1)	X (1.2)	P (1.3)	I (0.4)	I (0.48)	I (0.7)	H (2.5)	E (9.0)
F 1	-	-	+	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-
F 2	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
F 3	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
F 5	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
F 7	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
F 10	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-
F 11	-	-	-	-	-	-	-	-	-	+	S	-	+	-	-	-	-	-	-
F 14	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
F 16	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
F 21	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
F 22	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
F 23	-	-	-	-	-	-	-	-	-	+	F	-	+	-	+	-	-	-	-
F 28	-	-	-	-	-	-	+	+	-	-	F	-	+	-	-	-	-	-	-
F 33	-	-	-	-	-	-	-	-	-	+	S	-	+	-	-	-	-	-	-
F 39	-	-	-	-	-	-	+	-	-	-	S	-	+	-	-	-	-	-	-
S 2	-	-	+	-	-	-	-	-	-	+	S	-	+	-	-	-	-	-	-
S 3	-	-	+	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-
S 4	-	+	-	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-
S 5	-	-	-	-	-	-	-	-	-	-	F	-	+	-	+	-	-	-	-
S 6	-	+	-	-	-	-	-	+	-	-	F	-	+	-	-	-	-	-	-
S 7	-	+	-	-	-	-	-	-	-	+	F	+	+	-	-	+	-	-	-
S 13	+	-	-	-	-	-	-	-	-	+	F	+	+	-	-	+	-	-	-
S 18	-	+	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
S 24	-	-	+	-	-	-	-	-	-	+	F	+	+	-	-	+	-	-	-
S 26	+	-	-	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-
S 27	-	+	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
S 28	-	-	-	-	-	-	-	-	-	-	S	-	+	+	+	-	-	-	-
S 32	-	+	-	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-
S 34	-	-	-	-	-	-	-	-	-	+	F	+	+	-	+	-	-	-	-
S 39	-	-	-	-	-	-	+	+	-	-	F	-	+	-	-	-	-	-	-
								-	-	-	F	+	+	-	-	+	-	-	-

	E (-8.6)	X (-7.4)	B (-7.2)	I (0.35)	h (-6.9)	X (-5.3)	H (-3.7)	H (-3.0)	I (1.5)	D (o.2)	A	P (1.1)	X (1.2)	P (1.3)	I (0.4)	I (0.48)	I (0.7)	H (2.5)	E (9.0)
J 1	-	-	-	-	-	-	+	-	-	-	F	-	+	-	-	-	-	-	-
J 3	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-
J 4	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-
J 6	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
J 7	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-
J 8	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	+	-	-
J 11	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-
J 12	-	-	-	-	-	-	-	-	-	+	F	-	+	+	-	-	-	-	-
J 14	-	-	+	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-
J 16	-	-	+	-	-	-	-	-	-	-	S	-	+	+	+	-	-	-	-
J 21	-	-	-	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-
J 26	-	-	+	-	-	-	+	-	-	-	F	-	+	-	-	-	-	-	-
J 30	-	-	+	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-
J 36	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-
J 42	-	-	+	-	-	-	+	-	-	-	F r	-	+	-	-	-	-	-	-
D 2	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
D 4	+	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
D 6	-	-	-	-	-	-	-	+	-	-	F	-	+	-	-	-	-	-	-
D 7	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
D 10	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
D 11	+	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
D 12	-	-	-	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
D 14	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-
D 15	-	-	+	-	-	-	-	-	-	+	F	-	+	-	-	-	-	-	-
D 16	-	-	-	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-
D 17	-	-	-	-	-	-	-	+	-	-	F	-	+	-	-	-	-	-	-
D 20	-	+	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-
D 22	+	-	-	-	-	-	-	-	-	-	F	-	+	-	+	-	-	-	+
D 23	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-
D 30	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	+

Table 3.3.2 Restriction endonuclease variation in the *Adh* gene region of the Australian natural populations of *Drosophila melanogaster*. All the abreviations are the same as in table 3.3.1. Populations are C-Cygnet, T-Tamar, C.Y.-Chateau Yarriya, Al-All Saints, Ar-Araluen, Ch-Coffs Harbour, Cd-Cardwell.

	X (-8.0)	X (-7.4)	B (-7.2)	I (0.28)	h (-6.9)	X (-5.3)	H (-3.7)	H (-3.0)	D (0.2)	I (5.0)	A	P (1.1)	Xh (1.2)	p (1.3)	x (1.4)	I (0.4)	I (0.7)	I (1.0)	I (3.0)	I (4.5)	B (3.9)	E (9.0
C 3	-	-	+	+	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-	-	-	-
C 5	-	-	+	-	-	-	-	+	+	-	F	-	+	-	-	-	-	-	-	-	-	-
C 8	-	-	-	-	-	-	-	-	-	-	S	-	-	+	-	-	-	-	-	-	-	-
C 11	-	-	+	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-	-	-	+
C 19	-	-	+	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-	-	-	-
C 20	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-	-	-	-
C 25	-	-	+	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-	-	-	-
C 28	-	-	-	-	+	-	-	-	-	-	S	-	+	-	-	-	-	-	-	-	-	+
C 29	-	-	-	-	-	-	-	+	+	-	F	-	+	-	-	-	-	-	-	-	-	-
C 33	-	-	-	-	-	-	-	-	+	-	F	-	+	-	-	+	-	-	-	-	-	-
C 35	-	-	-	-	-	-	-	+	+	-	F	-	+	-	-	-	-	-	-	-	-	-
C 45	-	-	-	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-	-	-	-
C 51	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-	-	-	-
C 61	-	-	+	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	+
C 72	-	-	-	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-	-	-	-
T 1	-	-	-	-	+	-	-	-	-	-	S	-	+	+	-	+	-	-	-	-	+	-
T 12	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-	-	-	-
T 17	-	-	+	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-	-	+	-
T 19	-	-	+	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	+
T 29	-	-	-	+	+	-	-	-	-	-	F	-	+	-	-	-	-	-	-	-	-	+
T 30	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-	-	-	-
T 32	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-	-	-	+
T 33	-	-	+	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-	-	-	-
T 44	-	-	-	-	-	-	-	-	-	-	F	+	+	-	-	+	-	-	-	-	-	+
T 50	-	-	+	-	-	-	-	-	-	-	S	-	+	-	-	-	-	-	-	-	-	-
T 54	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-	-	-	-
T 56	-	-	+	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-	-	-	-
T 60	-	-	-	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-	-	-	-
T 73	-	-	+	-	-	-	-	-	-	-	F	-	+	-	-	-	-	-	-	-	-	-

	X (-8.0)	X (-7.4)	B (-7.2)	I (0.28)	h (-6.9)	X (-5.3)	H (-3.7)	H (-3.0)	D (0.2)	I (5.0)	A	P (1.1)	Xh (1.2)	p (1.3)	E (1.4)	I (0.4)	I (0.7)	I (1.0)	I (3.0)	I (4.5)	B (3.9)	E (9.0
C.Y.1	–	+	+	–	–	–	–	+	–	–	S	–	+	+	–	+	–	–	–	–	–	–
C.Y.2	+	–	+	–	–	–	–	+	–	–	S	–	–	+	–	–	–	+	–	–	–	–
C.Y.3	–	–	+	–	–	–	–	–	–	–	S	–	+	–	–	–	–	–	–	–	–	–
C.Y.4	+	–	+	–	–	–	–	+	–	–	S	–	–	+	–	–	–	+	–	–	–	–
C.Y.5	–	–	+	–	–	–	–	+	–	–	S	–	+	–	–	+	–	–	–	–	–	–
C.Y.6	–	+	+	–	–	–	–	–	–	–	S	–	–	+	–	–	–	–	–	–	–	–
C.Y.7	–	–	–	–	–	–	–	+	–	–	F	–	+	–	–	+	–	–	–	–	–	–
C.Y.8	–	–	–	–	–	–	–	–	–	–	F	–	–	–	–	–	–	–	–	–	–	–
C.Y.9	–	–	+	–	–	–	–	+	–	–	F	–	+	–	–	–	–	–	–	–	–	+
C.Y.10	–	–	–	–	–	–	+	–	+	–	F	–	+	–	–	–	–	–	–	–	–	–
C.Y.11	–	+	–	–	–	–	+	–	–	–	F	–	+	+	–	+	–	–	–	–	–	–
C.Y.12	–	–	+	–	–	–	–	–	+	–	F	–	+	–	–	–	–	–	–	–	–	–
C.Y.13	–	–	+	–	–	–	+	–	–	–	F	–	–	–	–	–	–	–	–	–	–	–
C.Y.14	–	–	–	–	–	–	+	–	–	–	F	–	+	–	–	–	–	–	–	–	–	–
C.Y.15	–	–	–	–	–	–	–	–	+	–	F	–	+	–	–	–	–	–	–	–	–	–
Al 1	–	+	+	+	–	–	–	–	–	–	S	–	+	+	–	+	–	–	–	–	–	–
Al 2	–	–	+	–	–	–	+	–	–	–	S	–	+	–	–	–	–	–	–	–	–	–
Al 3	–	+	+	–	–	+	–	–	–	–	S	–	+	+	–	+	–	–	–	–	–	–
Al 4	–	–	+	–	–	–	–	–	–	–	F	+	+	–	–	–	–	–	–	–	–	–
Al 5	–	+	–	–	–	–	–	–	–	–	F	–	+	–	–	+	–	–	–	–	–	–
Al 6	–	–	–	–	–	–	–	–	–	–	F	–	+	–	–	–	–	–	–	–	–	–
Al 7	–	–	+	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–	–	–	–
Al 8	–	–	–	–	–	–	–	+	+	–	F	–	+	–	–	–	–	–	–	–	–	–
Al 9	–	–	–	–	–	–	–	–	–	–	F	–	+	–	–	–	–	–	–	–	–	+
Al 10	–	–	–	–	–	–	–	–	–	–	F	+	+	–	–	+	–	–	–	–	–	–
Al 11	–	+	–	–	–	–	–	–	–	–	F	–	+	–	–	–	–	–	–	–	–	–
Al 12	–	–	+	–	–	–	–	–	–	–	F	–	+	–	–	–	–	–	–	–	–	–
Al 13	–	–	–	–	–	–	–	–	–	+	F	–	+	–	–	–	–	–	–	–	–	–
Al 14	–	–	–	–	–	–	+	–	+	–	F	–	+	–	–	–	–	–	–	–	–	+
Al 15	–	–	–	–	–	–	–	–	–	–	F	–	+	–	–	–	–	–	–	–	–	–

	X (−8.0)	X (−7.4)	B (−7.2)	I (0.28)	h (−6.9)	X (−5.3)	H (−3.7)	H (−3.0)	D (0.2)	I (5.0)	A	P (1.1)	Xh (1.2)	P (1.3)	E (1.4)	I (0.4)	I (0.7)	I (1.0)	I (3.0)	I (4.5)	B (3.9)	E (9.0
Ar 1	−	−	+	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−
Ar 2	−	−	+	−	−	−	−	−	−	−	S	−	+	−	−	−	−	−	−	−	−	+
Ar 3	−	−	−	−	−	−	−	−	−	−	S	−	+	−	−	−	−	−	−	−	−	+
Ar 4	−	−	+	−	−	−	−	−	−	−	S	+	+	−	−	+	−	−	−	−	−	−
Ar 5	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−
Ar 6	−	−	+	−	−	−	−	−	−	−	S	−	+	−	−	−	−	−	−	−	−	−
Ar 7	−	−	+	−	−	−	−	−	−	−	F	−	+	−	−	−	−	−	−	−	−	+
Ar 8	−	+	−	−	−	−	−	−	−	−	F	−	+	+	−	+	−	−	−	−	−	−
Ar 9	−	−	−	−	−	−	−	−	−	−	F	−	+	−	−	−	−	−	−	−	−	+
Ar 10	−	−	−	−	−	−	−	+	+	−	F	−	+	−	−	−	−	−	−	−	−	−
Ar 11	−	−	−	−	−	−	−	+	+	−	F	−	+	−	−	−	−	−	−	−	−	−
Ar 12	−	−	−	−	−	−	−	−	−	−	F	−	+	−	−	−	−	−	−	−	−	−
Ar 13	−	−	−	−	−	−	−	−	−	−	F	+	+	−	−	−	−	−	+	−	−	−
Ar 14	−	−	−	−	−	−	−	−	−	−	F	−	+	−	−	−	−	−	−	−	−	+
Ar 15	−	−	−	−	−	−	−	−	−	−	F	−	+	−	−	−	−	−	−	−	−	−
Ch 1	−	−	+	−	+	−	−	+	−	−	S	+	+	−	−	−	−	−	−	−	−	+
Ch 2	−	−	+	−	−	−	−	+	−	−	S	−	−	−	−	+	−	−	−	+	−	−
Ch 3	−	−	+	−	+	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−
Ch 4	−	−	+	−	−	−	−	+	−	−	S	+	+	−	−	+	−	−	−	−	−	+
Ch 5	−	−	−	−	−	−	−	+	−	−	F	−	+	−	−	−	−	−	−	−	−	−
Ch 6	−	−	−	−	−	−	−	−	+	−	F	−	+	−	−	−	−	−	−	−	−	−
Ch 7	−	−	+	−	−	−	−	+	+	−	F	−	+	−	−	−	−	−	−	−	−	−
Ch 8	−	−	+	−	−	−	−	+	−	−	S	−	+	−	−	−	−	−	−	−	−	−
Ch 9	−	−	+	−	−	−	−	+	−	−	F	−	+	−	−	−	−	−	−	+	−	−
Ch 10	−	−	+	−	−	−	−	+	−	−	S	+	+	−	−	−	−	−	−	−	−	−
Ch 11	−	−	+	−	−	−	−	−	−	−	F	−	+	−	−	−	−	−	−	−	−	−
Ch 12	−	−	+	−	−	−	−	−	−	−	S	−	+	−	−	−	−	−	−	−	−	−
Ch 13	−	−	+	−	−	−	−	−	−	−	F	−	+	+	−	−	−	−	−	−	−	−
Ch 14	−	−	−	−	−	−	−	−	+	−	null	+	+	−	+	−	−	−	−	−	−	+

	X (−8.0)	X (−7.4)	B (−7.2)	I (0.28)	h (−6.9)	X (−5.3)	H (−3.7)	H (−3.0)	D (0.2)	I (5.0)	A	P (1.1)	Xh (1.2)	P (1.3)	E (1.4)	I (0.4)	I (0.7)	I (1.0)	I (3.0)	I (4.5)	B (3.9)	E (9.0
Cd 1	−	−	−	−	−	−	+	−	+	−	S	−	−	−	−	−	−	−	−	−	−	−
Cd 2	−	−	+	−	−	−	−	−	−	−	S	−	+	−	−	−	−	−	−	−	−	−
Cd 3	−	−	+	−	−	−	−	−	−	−	S	+	+	−	−	+	−	−	−	−	−	−
Cd 4	−	−	+	−	−	−	−	+	−	−	S	−	+	−	−	−	−	−	−	−	−	+
Cd 5	−	−	+	−	−	−	−	+	−	−	S	−	−	+	−	−	+	−	−	−	−	−
Cd 6	−	−	+	−	+	−	+	−	+	−	S	−	+	−	−	−	−	−	−	−	−	−
Cd 7	−	−	+	−	−	−	−	+	−	−	S	−	−	−	−	−	−	−	−	−	−	−
Cd 8	−	−	+	−	−	−	−	+	−	−	S	−	−	−	−	−	−	−	−	−	−	+
Cd 9	−	−	−	−	−	−	−	−	−	−	F	−	+	−	−	−	−	−	−	−	−	−
Cd 10	−	−	−	−	−	−	+	+	−	−	F	−	−	−	−	−	−	−	−	−	−	−
Cd 11	−	−	−	−	−	−	−	−	−	−	F	−	−	−	−	−	−	−	−	−	−	−
Cd 12	−	−	+	−	−	−	+	+	−	−	F	−	+	−	−	−	−	−	−	−	−	−
Cd 13	−	−	−	−	−	−	−	−	−	−	F	−	−	−	−	−	−	−	−	−	−	−
Cd 15	−	−	−	−	+	−	−	−	−	−	F	−	+	−	−	−	−	−	−	−	−	−
Cd 16	−	−	−	−	−	−	−	−	−	−	F	−	+	+	−	−	−	−	−	+	+	+

Table 3.3.3 The frequencies of D(0.2) in Chinese populations and their regression on latitude.

Population	Latitude	frequency of D(0.2)
Haikou	$20^{\circ}02'$	0.8
Guangzhou	$23^{\circ}08'$	0.87
Fuzhou	$26^{\circ}05'$	0.73
Shanghai	$31^{\circ}10'$	0.4
Jinan	37°	0.13
Dalian	$38^{\circ}54'$	0.4

least-squares regression line $Y = 1.547 - 0.0338X$

correlation coefficent $-.89^{**}$ $(p < 0.01)$

table 3.3.4 Estimates of the proportion of polymorphic nucleotides, P, overall heterozygosity, h (Nei and Tajima ,1981) and estimated heterozygosity per nucleotide pair, θ (Ewens, Spielman and Harris, 1981). Standard errors are given in parentheses and that for θ assumes free recombination. N is the number of chromosome extracted from each population.

Populations	latitude	n	P	h	θ
Cygnet	43°09′S	15	0.017(0.007)	0.95(0.014)	0.006(0.0024)
Tamar	41°02′S	15	0.019(0.007)	0.86(0.057)	0.007(0.0026)
Chateau Yarrinya	37°36′S	15	0.022(0.011)	0.99(0.0014)	0.0082(0.0039)
All Saints	36°03′S	15	0.024(0.0081)	0.95(0.014)	0.009(0.0048)
Araluen	35°39′S	15	0.020(0.0076)	0.96(0.010)	0.0074(0.0028)
Coffs Harbour	30°18′S	14	0.024(0.0081)	1.00(--)	0.009(0.003)
Cardwell	30°18′S	15	0.024(0.0081)	0.98(0.0023)	0.009(0.003)
overall Australian populations		104	0.021(0.0058)	0.96(0.01)	0.008(0.0022)
Haikou	20°02′N	15	0.017(0.007)	0.94(0.018)	0.0064(0.0026)
Guangzhou	23°08′N	15	0.012(0.0062)	0.94(0.018)	0.0046(0.0023)
Fuzhou	26°05′N	15	0.0096(0.0055)	0.83(0.07)	0.0036(0.002)
Shanghai	31°10′N	15	0.019 (0.007)	0.89(0.04)	0.007(0.0026)
Jinan	37° N	15	0.012(0.0062)	0.90(0.037)	0.0046(0.0023)
Dalian	38°54′N	15	0.015(0.0067)	0.76(0.13)	0.0055(0.0025)
overall Chinese populations		90	0.014(0.0042)	0.88(0.047)	0.005(0.0015)
overall		194	0.03(0.0029)	0.94(0.018)	0.0065(0.0005)

Table 3.3.5.a Restriction map haplotype diversity in the *Adh* region in the Chinese populations. The numbers of different haplotypes for each category are given in parentheses.

variation considered	all chromosomes	Adh^F	Adh^S	$Adh^{FCH.D}$
restriction site	0.90(28)	0.78(14)	0.80(10)	0.64(4)
sequence length	0.86(14)	0.67(7)	0.71(4)	0.46(3)
all variation	0.96(48)	0.90(31)	0.92(13)	0.64(4)

Table 3.3.5.b *Adh* restriction map haplotype diversity in the Australian populations.

variation considered	all chromosomes	Adh^F	Adh^S
restriction site	0.95(48)	0.90(19)	0.97(28)
sequence length	0.79(16)	0.58(8)	0.54(7)
all variation	0.97(59)	0.93(28)	0.97(30)

Table 3.3.6 Significant gametic disequilibria in the Australian and Chinese populations. The abbreviations, I and D represent insertions and deletions, their size(kb) are given in parentheses.

Populations	Significant gametic disequilibria
Australia	
Cygnet	HindIII(-3.0):Adh*, HindIII(-3.0):D(0.2)** Adh:D(0.2)*
Tamar	BamHI(-7.2):Adh**, HpaI(-6.9):BamH(3.9)**, PstI(1.1):I(0.4)**, XhoI(1.2):EcoRI(9.0)*
Chateau Yarrinya	HindIII(-3.0):HindIII(-3.7)*, HindIII(-3.0):D(0.2)*, BamHI(-7.2):Adh**, I(1.0):XbaI(-8.0)**, XbaI(-7.4):I(0.4)**
All saints	BamHI(-7.2):Adh**, XbaI(-5.2):XbaI(-7.4)*, XbaI(-5.2):PstI(1.3)*, I(0.4):XbaI(-7.4)**, I(0.4):XbaI(-5.2)*, I(0.4):PstI(1.3)*
Araluen	BamHI(-7.2):Adh**, HindIII(-3.0):D(0.2)**
Coffsharbour	HpaI(-6.9):I(0.4)**, I(0.4):PstI(1.1)*, HpaI(-6.9):BamHI(3.9)**, I(0.4):BamHI(3.9)*
Cardwell	BamHI(-7.2):Adh**, HindIII(-3.7):D(0.2)**,
China	
Haikou	D(0.2):EcoRI(9.0)*, PstI(1.3):I(0.4)*
Guangzhou	BamHI(-7.2):Adh*
Fuzhou	None
Shanghai	BamHI(-7.2):Adh**, D(0.2):PstI(1.1)* I(0.48):Xba(-7.4)**, I(0.48):BamHI(-7.2)* I(0.48):PstI(1.3)**
Jinan	BamHI(-7.2):Adh**
Dalian	None

* $p<0.05$, ** $p<0.01$.

Table 3.4.1 A comparison of of the estimated proportion of polymorphic nucleotides (P), overall heterozygosity (h) and heterozygosity per nucleotide pair (θ) between Australian and chinese populations and in populations previously surveyed.

populations	p	h	θ	authors
Australia	0.021	0.96	0.008	
China	0.014	0.88	0.005	
North America	-	-	0.006	Langley, *et al.*, 1982
Texas	0.012	0.86	0.004	Birley, 1984
Chateau Tahbilk	0.017	0.94	0.005	Cross and Birley, 1986
Groningen	0.025	0.665	0.007	Cross and Birley, 1986
North American	-	0.97	0.006	Aquadro, *et al.*, 1986

Data from Australian and Chinese populations are calculated as same as table 3.3.4. "-" denotes not available.

Figure 3.1.1 The location of the seven Australian populations of *Drosophila melanogaster* sampled for estimating restriction endonuclease variation.

Figure 3.1.1


Cardwell
Coffs Harbour
Araluen
All Saints
Chateau Yarrinya
N
Tamar
Cygnet

Figure 3.1.2 The location of the six Chinese populations of *Drosophila melanogaster* sampled for estimating restriction endonuclease variation.

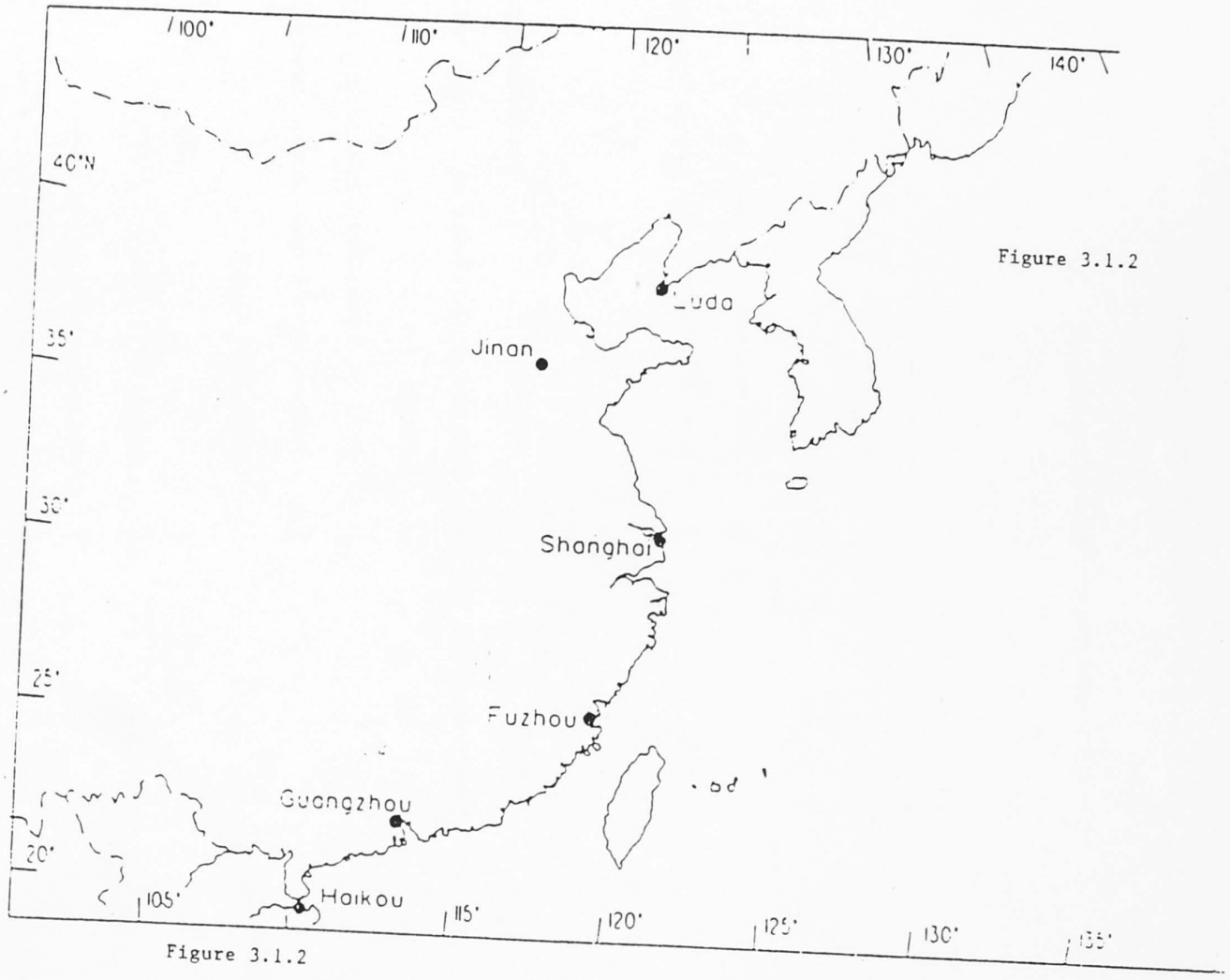


Figure 3.1.2

Figure 3.1.2

Figure 3.2.1 The breeding programme used to produce single second chromosome lines.

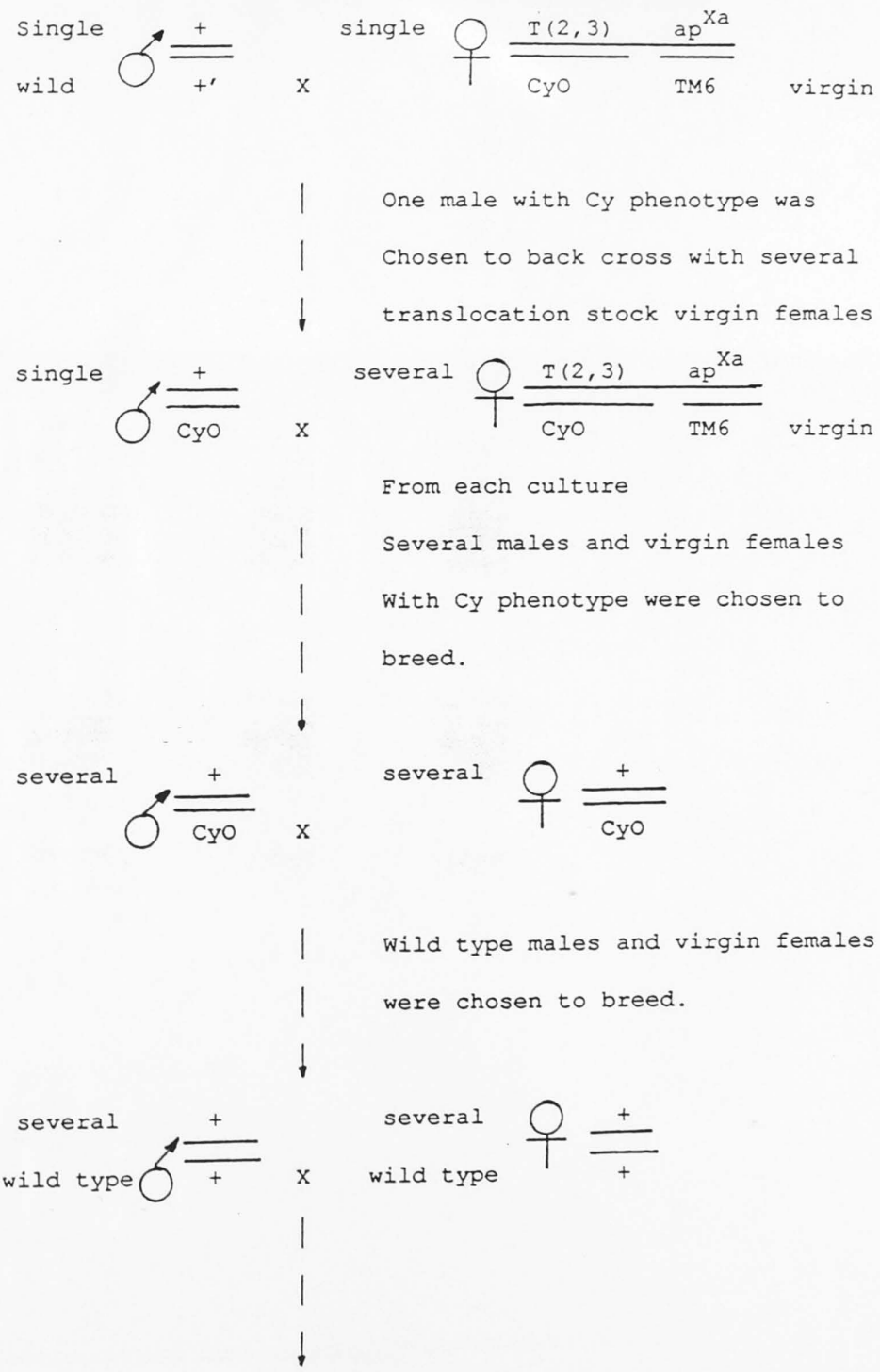

Single
wild
+
+'
X
single
T(2,3)
ap^Xa
CyO
TM6
virgin
One male with Cy phenotype was
Chosen to back cross with several
translocation stock virgin females
single
CyO
several
From each culture
Several males and virgin females
With Cy phenotype were chosen to
breed.
several
wild type
Wild type males and virgin females
were chosen to breed.
maintained as
single second chromosome line

Figure 3.2.1

Figure 3.2.2 Plasmid sAF2 was digested by restriction endonucleases and electrophoresis was carried out in 1% agarose gel. Lanes (1), (5),(9) lamda DNA cut by *Hin*dIII as a marker; and in the following lanes sAF2 was cut by (2) *Sal*I; (3)*Sac*I; (4)*Hpa*I; (6)*Bam*HI; (7)*Hin*dIII; (8)*Eco*RI.

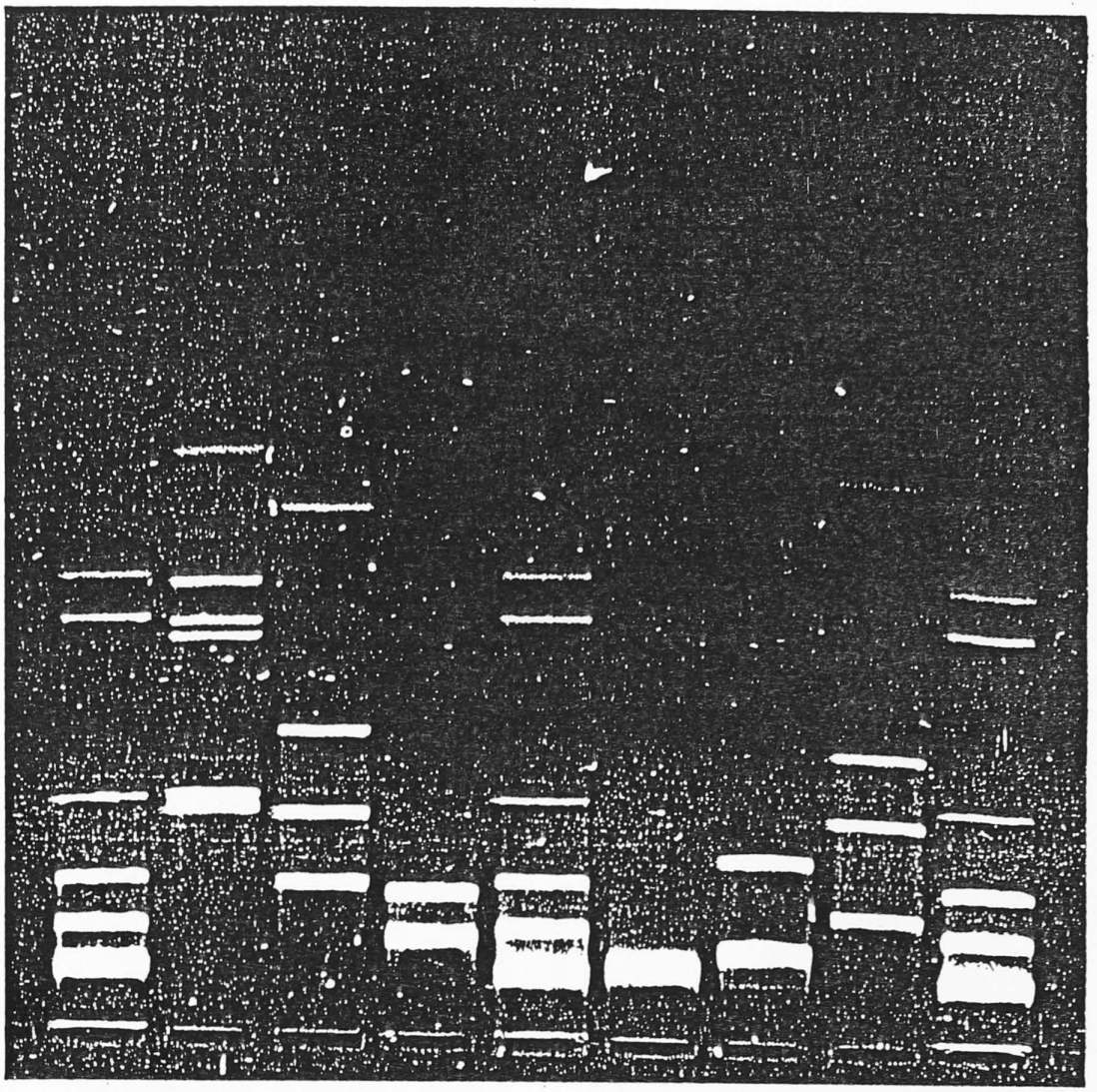


Figure 3.2.2

Figure 3.2.3 Restriction map of the *Adh* gene region taken from Goldberg (1980) and checked by restiction endonuclease digestions in this study. Fragment sizes after digestion with eight restriction endonucleases and the region covered by the sAF2 (11.8kb) clone are indicated. The scale(kb) is oriented with 0.0 at the *Bam*HI site in the *Adh* coding region.

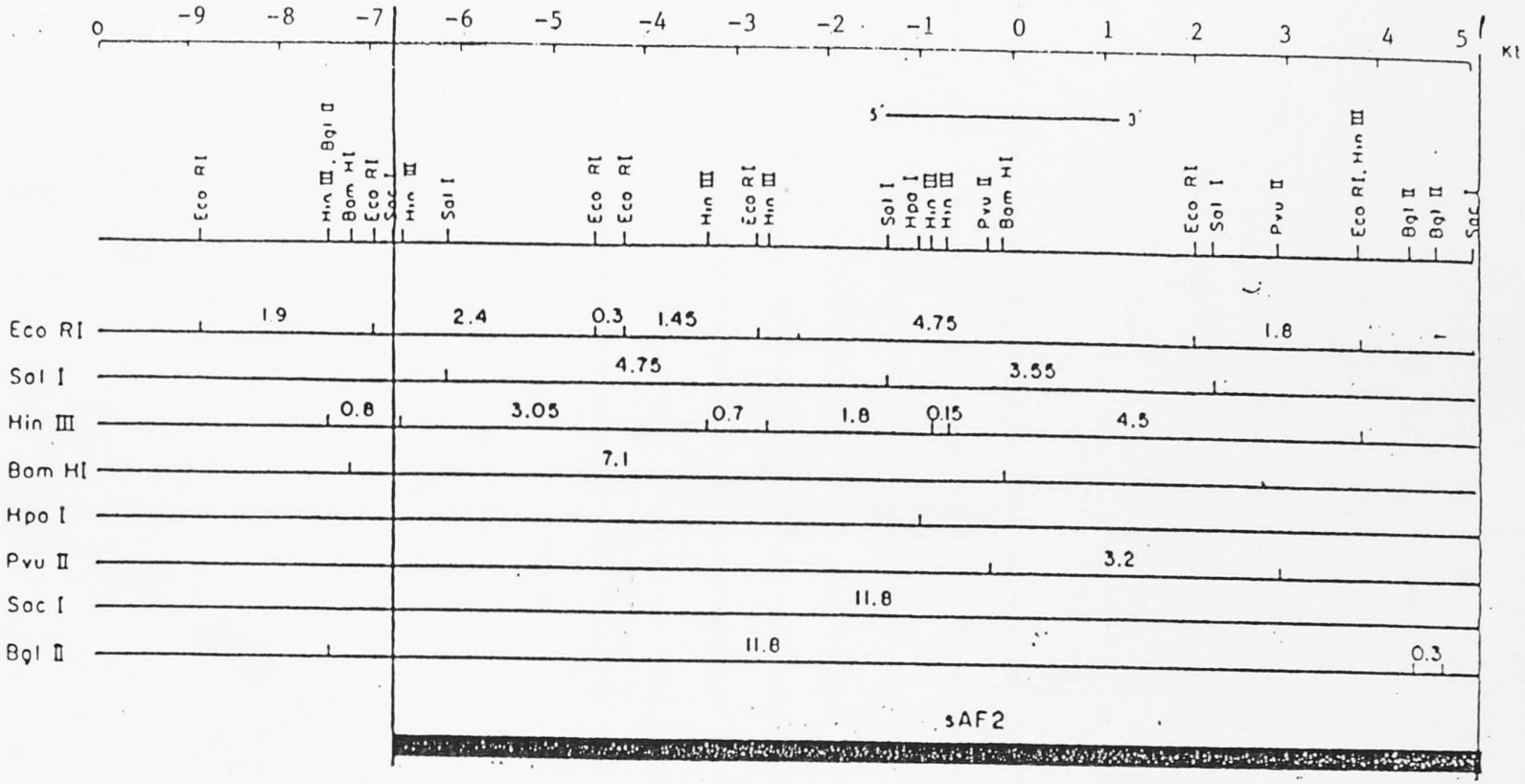

0 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 Kl
Eco RI
Hin III, Bgl II
Bam HI
Eco RI
Sac I
Hin III
Sal I
Eco RI
Eco RI
Hin III
Eco RI
Hin III
Sal I
Hpa I
Hin III
Hin III
Pvu II
Bam HI
Eco RI
Sal I
Pvu II
Eco RI, Hin III
Bgl II
Bgl II
Sac I
Eco RI
Sal I
Hin III
Bam HI
Hpa I
Pvu II
Soc I
Bgl II
1.9
2.4
0.3
1.45
4.75
1.8
4.75
3.55
0.8
3.05
0.7
1.8
0.15
4.5
7.1
3.2
11.8
11.8
0.3
sAF2

Figure 3.2.3

Figure 3.3.1.a Restriction endonuclease variation detected in the *Adh* gene region of chromosomes from Australian populations. The boundaries of the *Adh* transcription unit are indicated and the adult 5′ leader sequence and exons are shaded. Variable restriction endonuclease sites are indicated below the map; triangles represent insertions (pointing towards the map) and deletions (pointing away from the map). The estimated sizes (bp) are shown. The *Eco*RI(9.0) site was outside the probed region but could be scored unambigiously in all populations.

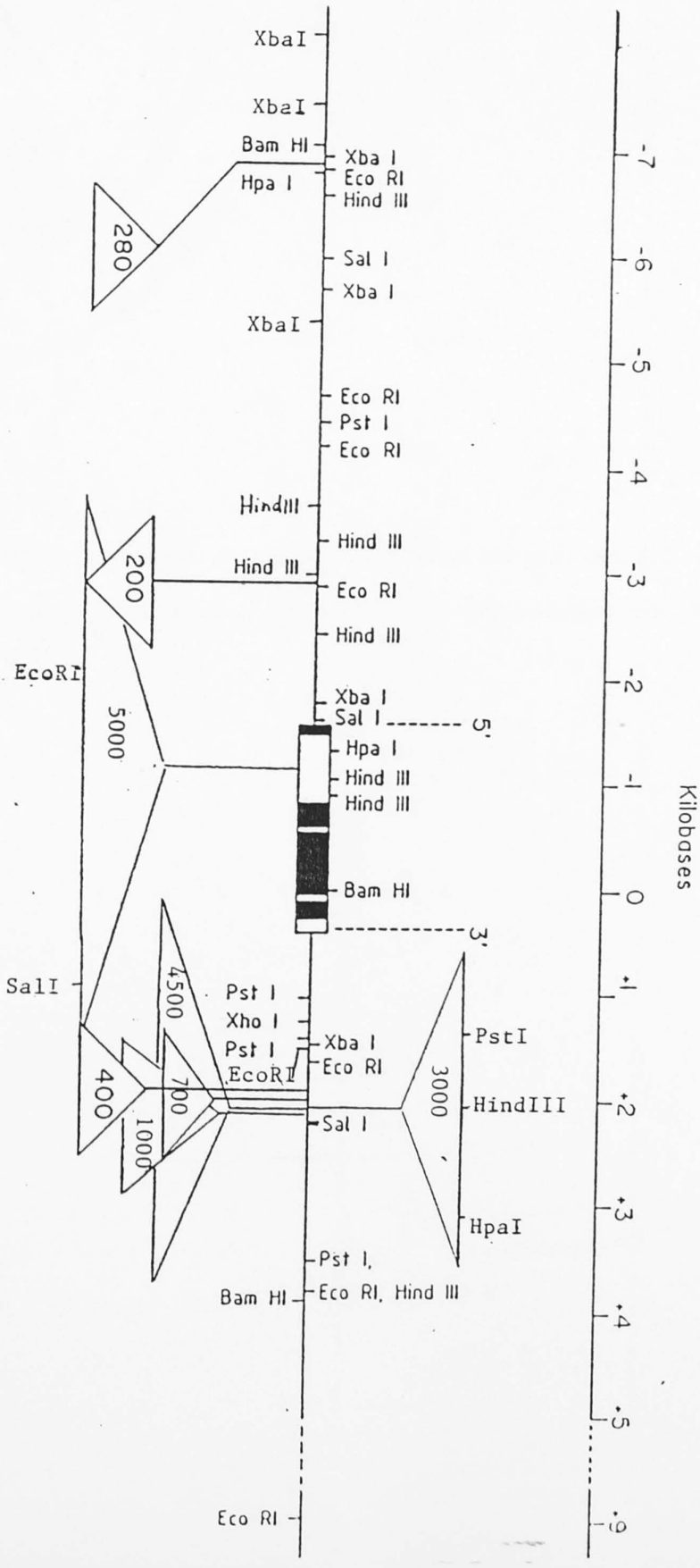

XbaI
XbaI
Bam HI
Xba I
Eco RI
Hpa I
Hind III
280
Sal I
Xba I
XbaI
Eco RI
Pst I
Eco RI
HindIII
Hind III
Hind III
200
Eco RI
Hind III
EcoRI
Xba I
Sal I
5'
5000
Hpa I
Hind III
Hind III
Bam HI
3'
SalI
4500
Pst I
Xho I
Pst I
Xba I
Eco RI
EcoRI
PstI
400
700
3000
HindIII
1000
Sal I
HpaI
Pst I,
Bam HI
Eco RI, Hind III
Eco RI
-7
-6
-5
-4
-3
-2
-1
0
+1
+2
+3
+4
+5
+6
Kilobases

Figure 3.3.1.a

Figure 3.3.1.b Restriction endonuclease map variation detected in the *Adh* gene region of chromosomes from six Chinese populations (as in Figure 3.3.1.a).

Figure 3.3.1.b

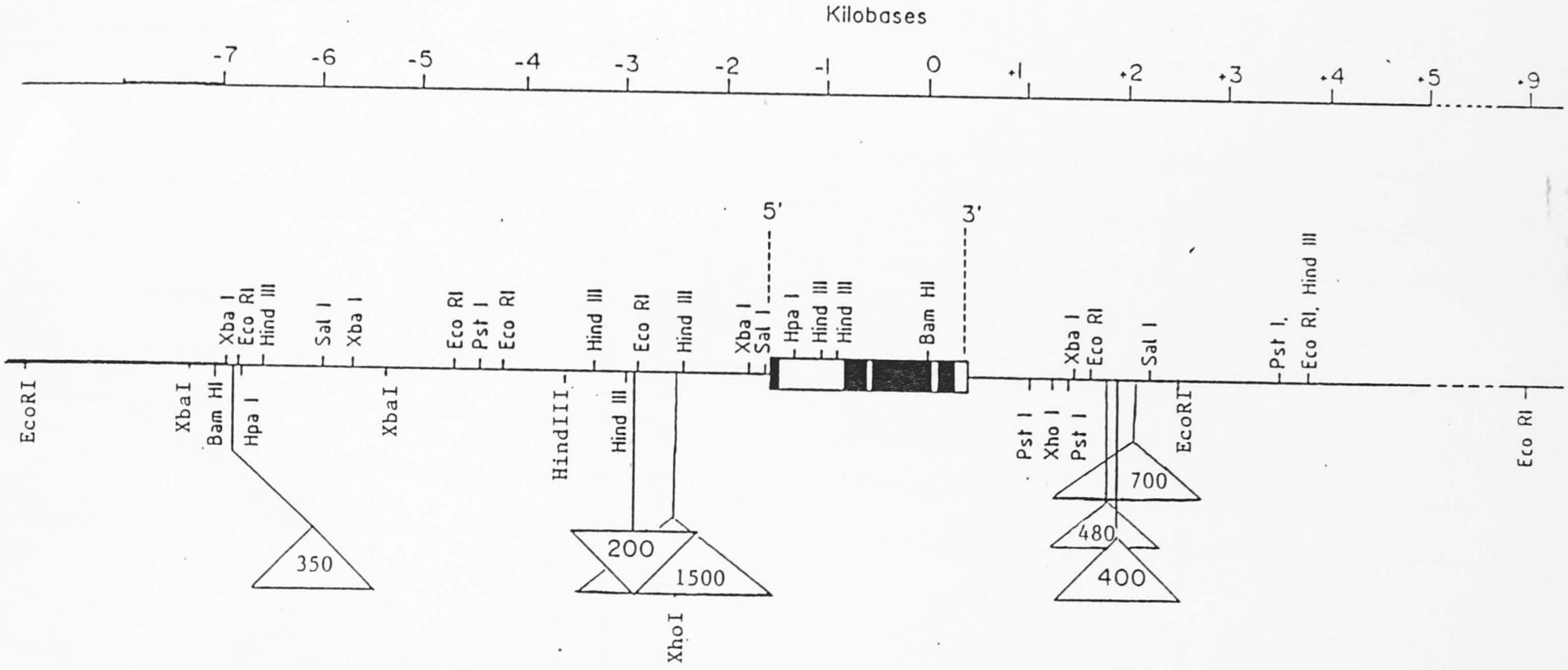

Kilobases
-7
-6
-5
-4
-3
-2
-1
0
+1
+2
+3
+4
+5
+9
5'
3'
EcoRI
XbaI
Bam HI
Hpa I
Xba I
Eco RI
Hind III
Sal I
Pst I
HindIII
Xho I
350
200
1500
700
480
400

Figure 3.3.2 Restriction map of the T40 line. The sizes of fragments cleavaged by eight restriction endonucleases are shown below the map. The regions which appear to be duplicated are indicated by a heavy line. The probed region extended to 16kb.

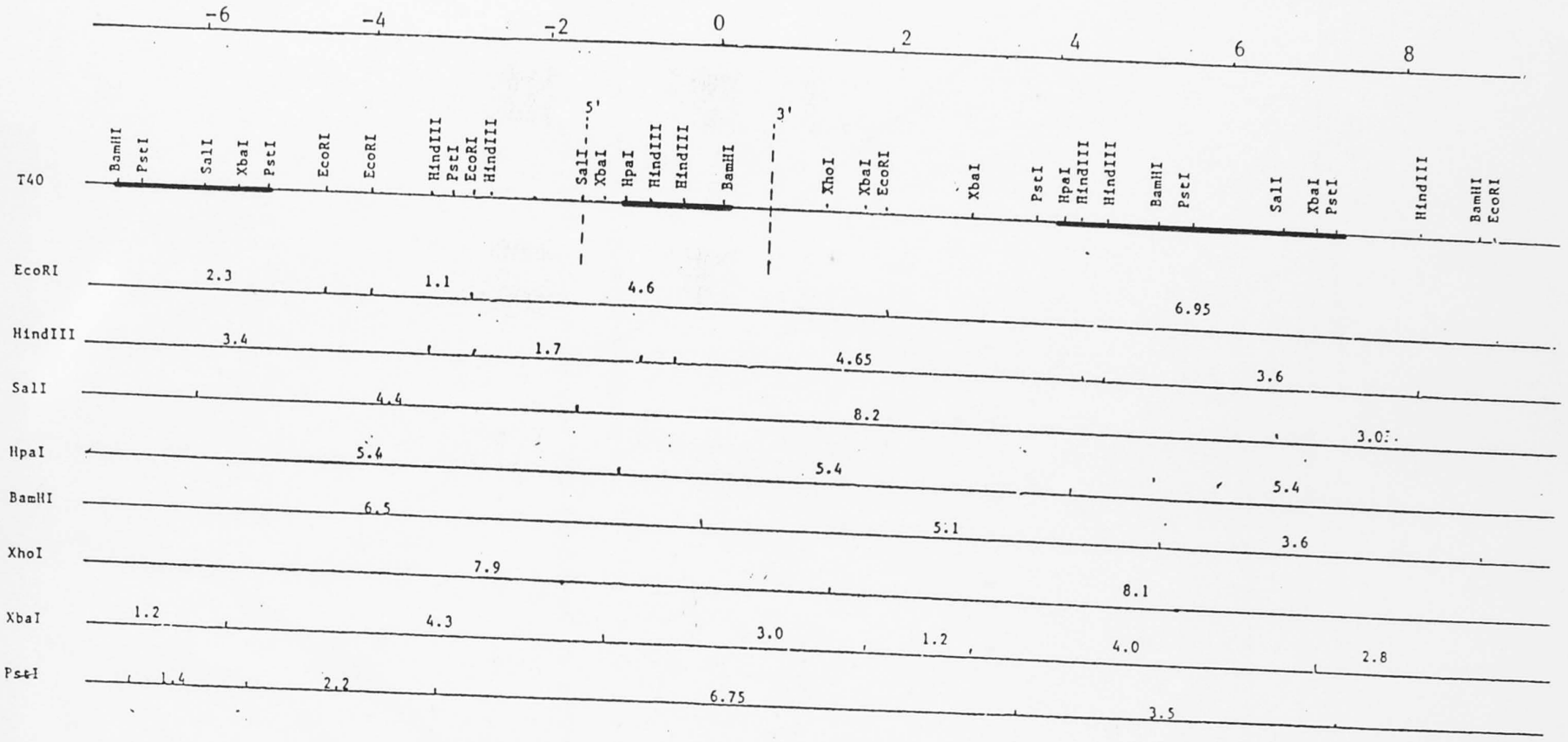

-6
-4
-2
0
2
4
6
8
T40
BamHI
PstI
SalI
XbaI
PstI
EcoRI
EcoRI
HindIII
PstI
EcoRI
HindIII
SalI
XbaI
5'
HpaI
HindIII
HindIII
BamHI
3'
XhoI
XbaI
EcoRI
XbaI
PstI
HpaI
HindIII
HindIII
BamHI
PstI
SalI
XbaI
PstI
HindIII
BamHI
EcoRI
EcoRI
2.3
1.1
4.6
6.95
HindIII
3.4
1.7
4.65
3.6
SalI
4.4
8.2
3.0
HpaI
5.4
5.4
5.4
BamHI
6.5
5.1
3.6
XhoI
7.9
8.1
XbaI
1.2
4.3
3.0
1.2
4.0
2.8
PstI
1.4
2.2
6.75
3.5

Figure 3.3.2

Figure 3.3.3 The relationship between the frequency of deletion 0.2kb and latitude in the Chinese populations.

Figure 3.3.3

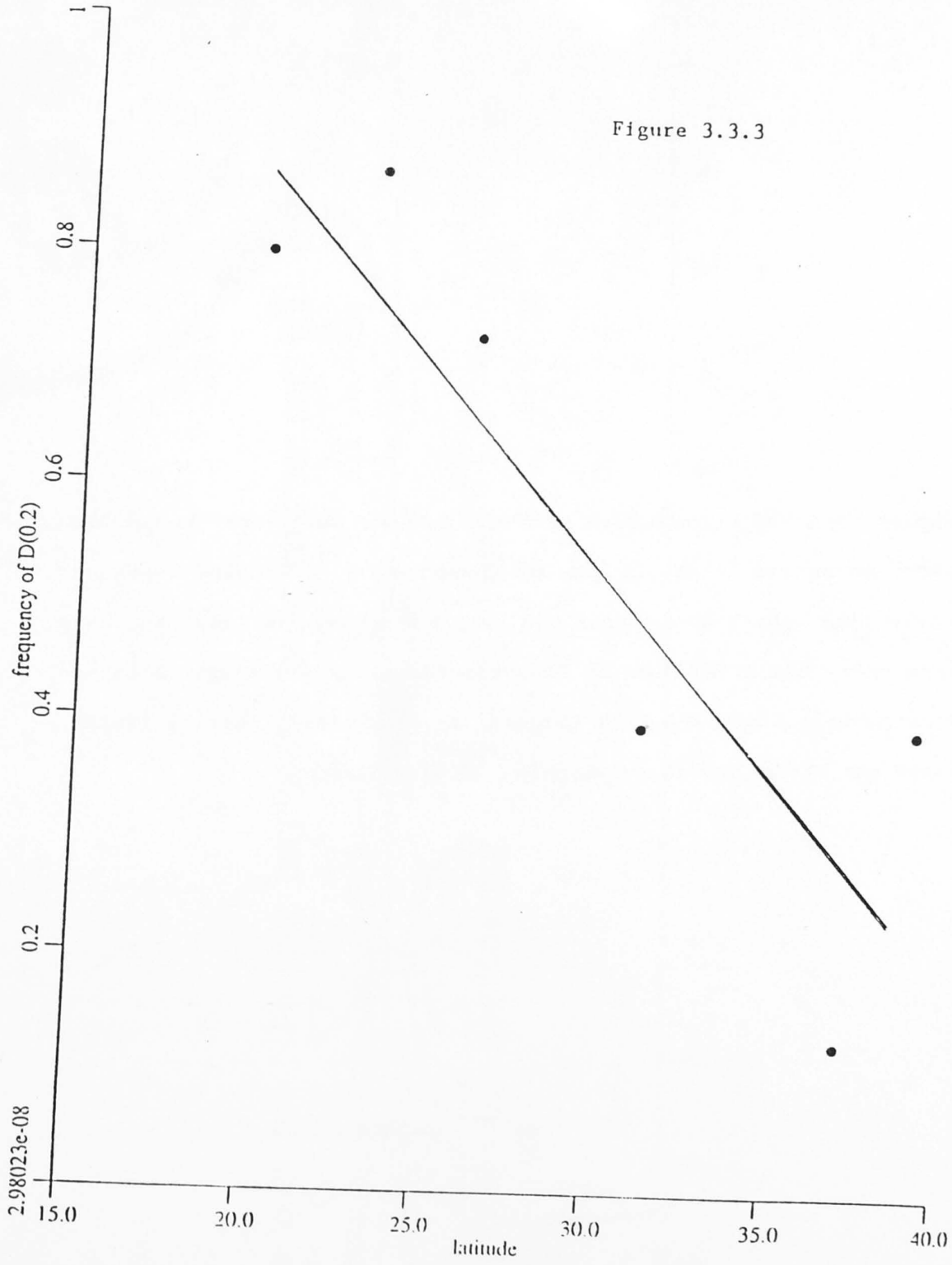

frequency of D(0.2)
1
0.8
0.6
0.4
0.2
2.98023e-08
15.0
20.0
25.0
30.0
35.0
40.0
latitude

Figure 3.4.1 The distribution of insertions and deletions (larger than 50bp) occurring in the 11.8kb *Adh* gene region. Triangles represent insertions (pointing towards the map) and deletions (pointing away from map), the sizes (bp) of insertion/deletion are shown. Data are from present study and from Langley, *et al.*, (1982), Birley (1984), Cross and Birley (1986) and Aquadro, *et al.*, (1986).

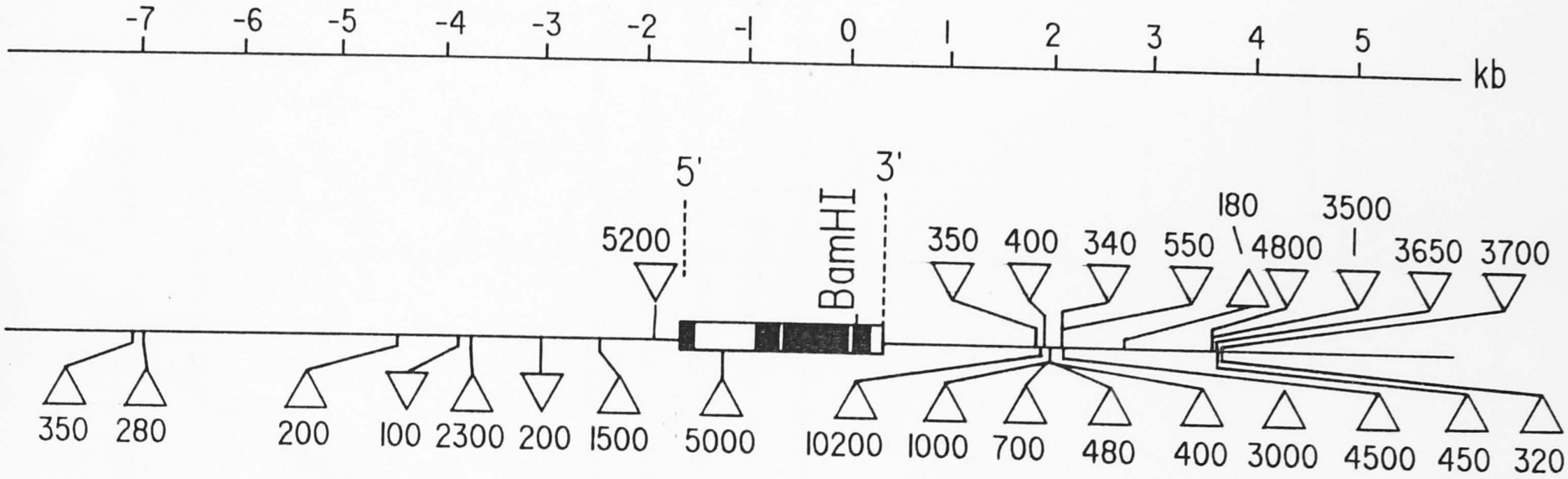
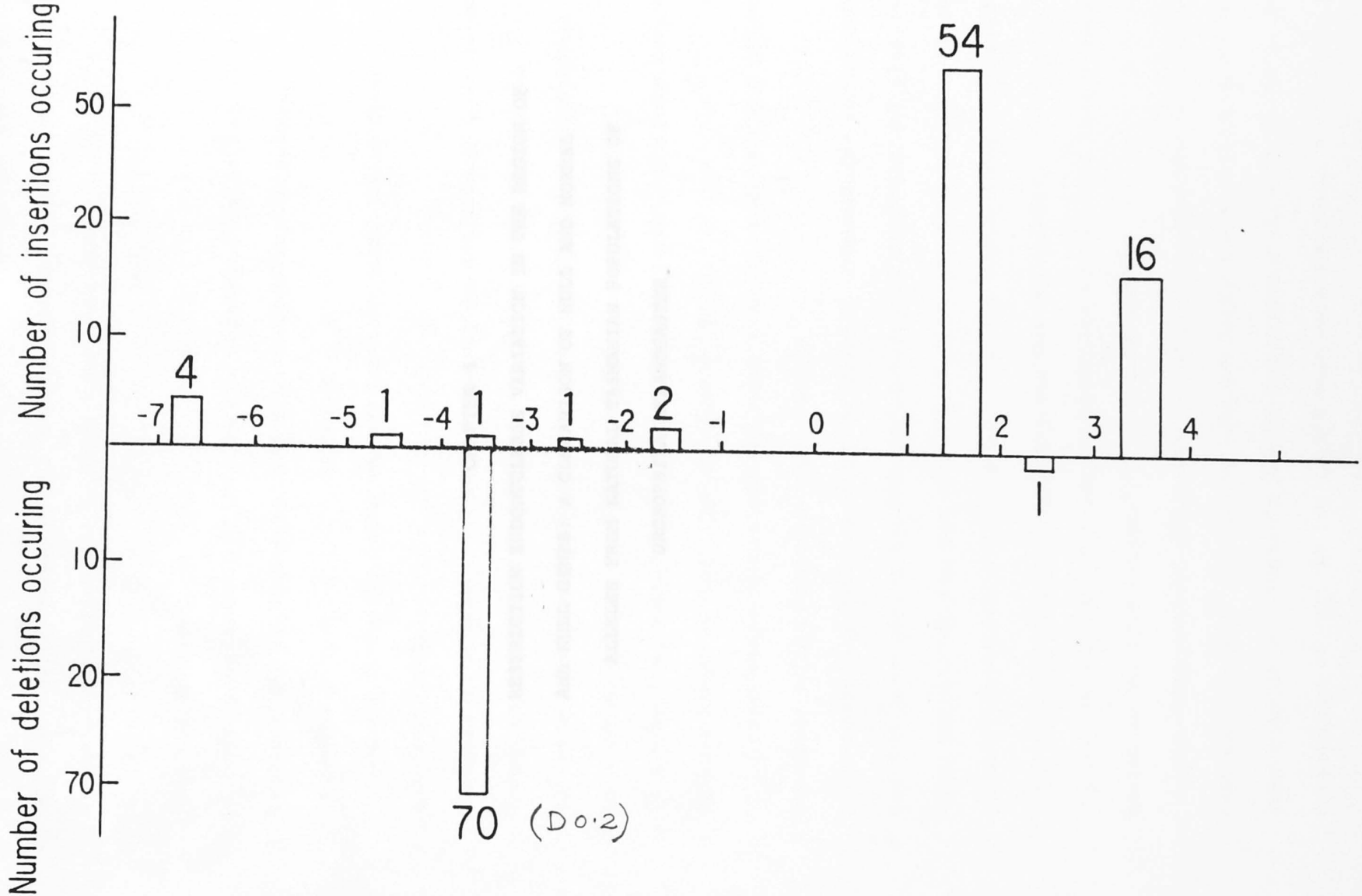

-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
kb
5'
3'
BamHI
5200
350
400
340
550
180
4800
3500
3650
3700
350
280
200
100
2300
200
1500
5000
10200
1000
700
480
400
3000
4500
450
320

Figure 3.4.2 Histogram for the distribution of insertions and deletions (bigger than 50bp) occurring in the 11.8kb region of *Adh* gene in natural populations of *Drosophila melanogaster*. The columns represent the number of insertions (above the scale) and deletions (below the scale) occurring in 335 lines investigated. Data sources are the same as in Figure 3.4.1.


Number of insertions occuring
Number of deletions occuring
50
20
10
10
20
70
4
1
1
1
2
54
1
16
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
70 (D0·2)

CHAPTER 4

RESTRICTION ENDONUCLEASE VARIATION IN THE REGION OF *Adh* NULL GENES: A COMPARISON OF NULL AND NORMAL ALLELES FROM NATURAL TASMANIAN POPULATIONS OF *DROSOPHILA MELANOGASTER*

Chapter 4 Restriction endonuclease variation in the region of *Adh* null genes: a comparison of null and normal alleles from natural Tasmanian populations of *Drosophila melanogaster*.

4.1 Introduction

The populations of *Drosophila melanogaster* on the island of Tasmania differ from populations on the Australian mainland in four respects:

(1)the *Adh* frequency cline appears to be reversed (Wilks *et al.*, 1980; Anderson, 1981).

(2)there is a relatively high frequency of *Adh* null alleles in some Tasmanian populations.

(3)there is a very low frequency of chromosome inversions (Knibb *et al.*, 1982).

(4)active *P* elements have not been found in Tasmanian populations and neither has male recombination (Boussy 1987).

These differences raised some questions about the genetic structure of the Tasmanian populations.:

(1)Is there any difference in restriction endonuclease variation in the *Adh* gene region between populations in Tasmania and elsewhere which may account for the reversed cline of *Adh* gene frequency in this island? Is there evidence for linkage disequilibrium between restriction endonuclease variants and the *Adh* allele?

(2) Do the *Adh* null alleles in Tasmanian populations have a common origin or multiple origins?

The frequency of *Adh* varies in different populations, with the frequency of Adh^F increasing with increasing distance from equator (see chapter 2). Tasmania covers latitudes from 41°S to 43°S. The frequencies of Adh^F in the island were expected to be higher than on

the Australian continent, and higher in the south of the island than in the north, but previous observations failed to support this prediction. The frequency of Adh^F in the Cygnet population in southern Tasmania (43°S) was 0.44, which is much lower than that in the south east of the Australian continent, for example it was 0.81 in Melbourne (37.7°S), 0.75 in Canberra (35.5°S) (Wilks *et al.*,1980; Anderson, 1981 and Oakeshott *et al.*, 1982). In the south of the island, the frequencies of Adh^F were 0.55, and 0.49 in two Cradoc populations (43.2°S); and in the north of the island the frequency of Adh^F was 0.71 at Pipers brook (41.1°S) (Anderson and Gibson, 1985). The cline in the island appears to be reversed and also the frequencies of Adh^F are much lower that expected when compared with populations at similar latitudes, such as Dalian population (China) (0.86)(chapter 2), or populations in northern France (0.95) (David *et al.*, 1989).

The frequency of *Adh* null alleles in natural populations of *Drosophila melanogaster* is usually very low. Voelker *et al.*, (1980) and Langley *et al.*, (1981) investigated 25 enzyme loci in a Raleigh (North Carolina) and in a London population of *Drosophila melanogaster*, and found null alleles at 13 enzyme loci in the Raleigh population, and at nine enzyme loci in the London population. The frequencies of these null alleles were usually less than 0.3%. A single *Adh* null allele was found in the Raleigh population, but on further investigation this was found to have low ADH activity (Ashburner, personal communication).

Null alleles at the alcohol dehydrogenase (*Adh*) locus in Tasmanian populations of *Drosophila melanogaster* are found at relatively high frequency (up to 3.9%) (Freeth and Gibson, 1985). It was shown that these null alleles remained in the populations over a number of years,

and this prompted questions about the origins of the alleles and their maintenance in natural populations.

The *Adh* null alleles were found in geographically separate populations in Tasmania, yet all the alleles investigated had similar biochemical properties. Freeth *et al.* (1986) analysed 23 *Adh* null alleles extracted from four Tasmania populations, Cygnet, Huonville, Pipers Brook, and Tamar. Their analyses showed that the null alleles did not produce any ADH activity, and of those tested , all were CRM (cross-reacting material) negative. The biochemical data did not provide any evidence for heterogeneity in properties between separately extracted *Adh* null alleles, although the homozygous viabilities of second chromosomes bearing the null alleles varied from lethal to normal (Freeth 1986).

The restriction endonuclease variation in the region of *Adh* gene of the *Adh* null alleles can be compared with a number of different normal alleles that are present in the same populations. Whilst the molecular identities of alleles with similar properties can be gauged from the DNA sequences, variation in the molecular landscape surrounding a locus can be assessed with restriction endonuclease mapping and used to derive the ancestry of specific gene mutations. This approach has been successfully applied in human populations to trace the origins of β-thalassaemia (Antinarakis *et al.*, 1982), sickle cell haemoglobin genes (Pagnier *et al.*, 1984), serum albumin variation (Murray *et al.*, 1984) and α-antitrypsin deficiency alleles (Cox *et al.*, 1985).

To provide some indication of the possible differences between Tasmanian populations and populations elsewhere, and the heterozygosity and relatedness of the *Adh* null alleles, 58 second chromosomes bearing *Adh* normal and null alleles extracted from three

Tasmanian populations of *Drosophila melanogaster* were mapped using an 11.8kb sAF2 probe which included the region of *Adh* gene. These data were used to test whether the molecular landscape surrounding the *Adh* null activity alleles differs from that of normal alleles in the same populations and to compare null alleles extracted from different populations, and in different years, to assess their molecular similarities.

4.2 Materials and Methods

4.2.1 Stocks

The 58 second chromosomes bearing *Adh* alleles used in these experiments were extracted from three of the Tasmanian populations of *Drosophila melanogaster* sampled in May, 1984 and 1985 (Freeth and Gibson, 1985). Two of the populations, Cygnet and Huonville (Avondale Farm) are about 13km apart in the south of the island and the third, Tamar (Marian's vineyard in the Tamar valley), is 300km to the north (refer to Figure 3.1.1).

The Cygnet and Tamar populations were sampled in 1984 by setting up single female lines (75 for Cygnet and 82 for Tamar) and extracting second chromosomes bearing normal *Adh* alleles (one from each line) using *CyO* (Lindsley and Grell, 1968) as a balancer (see Freeth and Gibson, 1985 and Chapter 3). The Huonville II population was sampled in 1985 by J.B. Gibson and A.V. Wilks, who extracted the second chromosomes by crossing a male from a single female line to females heterozygous for *CyO* and a deficiency covering the *Adh* locus, *Df(2L)64j*, (Lindsley and Grell, 1968), then backcrossing a single *+/Df(2L)64j* male to *CyO/Df(2L)64j* females to establish a *+/Df(2L)64j* line.

The null alleles were isolated by J.B. Gibson, A.L. Freeth and A.V. Wilks. The methods they used to detect putative *Adh* null alleles in the Cygnet and Tamar samples followed the technique of Voelker *et al.*, (1980) modified to avoid dysgenic phenomena (Freeth and Gibson, 1985). From the flies collected at Huonville II in 1985, 185 single female line were screened by classifying samples of their progenies into ADH electrophoretic phenotypes (see Freeth and Gibson, 1985). Abnormal segregation patterns in seven of the lines suggested the presence of null alleles and these were isolated. In addition two null alleles (H36 and H41) had been extracted from the Huonville II population in 1984. The frequencies of *Adh* null alleles in the three populations were 0.4 per cent (Cygnet,1984), 3.2 per cent (Huonville II, 1985) and 0.5 per cent (Tamar, 1984). The 12 null alleles used in this study (one from Cygnet, three from Tamar, two from Huonville II in 1984 and six in 1985) were isolated either by *CyO* as balancer or by exposing the F2 progenies of single female lines to 5% penten-3-ol vapour (Sofer and Hatkoff, 1972) and isolating the surviving flies, which are homozygous for an *Adh* null allele.

The numbers of normal *Adh* alleles investigated from each populations are given in tables 4.3.1, 4.3.2 and 4.3.3; the alleles were taken at random from those available from the chromosome extractions.

4.2.2 ADH electrophoresis

The electrophoretic ADH phenotypes of flies homozygous or hemizygous for extracted *Adh* alleles were assessed on cellulose acetate membranes stained for alcohol dehydrogenase activity as described by Lewis and Gibson (1978) (see chapter 2)

4.2.3 DNA extraction

DNA from the Cygnet and Tamar samples and from all the null alleles, was extracted from adult flies following the method of Miklos (1984) (see chapter 3). The Huonville II DNA samples were extracted by A.V. Wilks using the method of Chia *et al.* (1985): 50 adult flies were frozen in liquid nitrogen, then ground in a Eppendorf tube in 0.35ml of grinding buffer (10mM Tris-HCl pH7.5, 60mM NaCl, 10mM EDTA, 0.15mM spermidine, 0.15mM spermine and 5% (W/V) sucrose). After grinding 0.35ml of extraction buffer (0.2M Tris-HCl pH9.0, 30mM EDTA, 2% (W/V) SDS, 200µg proteinase K/ml and 5% sucrose) was added and mixed well. The mixture was incubated at 37°C in a water bath for 2 hours, then extracted with an equal volume of neutralized phenol and centrifuged at 10,000rpm for 5 minutes. The aqueous phase was transferred to a fresh Eppendorf tube and re-extracted with neutralized phenol and chloroform (1:1). The re-extracted aqueous phase was mixed with 2 volumes of cold ethanol in a fresh tube, placed at -20°C for 10 minutes then centrifuged at 10,000rpm for another 5 minutes. The pellet was seen in the bottom of the tube and washed with 75% ethanol, dried in vacuum for 7 minutes, then resuspend in 200µl of suspension buffer (10mM Tris-HCl pH8.0, 1mM EDTA) and boiled RNase was added to the concentration of 10µg/ml. The DNA solution was stored at -20°C until use. The concentration of the DNA was usually 50-100µg/ml.

4.2.4 Restriction endonuclease digests and electrophoresis of DNA

The eight hexanucleotide restriction endonucleases, conditions of digestions and electrophoresis of DNA used in the experiments described in this chapter were as used in chapter 3. The restriction digestions of Huonville II samples and the null alleles were carried out by J.B. Gibson and A.V. Wilks.

4.2.5 Hybridisation and construction of restriction maps.

These methods have been described in chapter 3.

4.3 Results

The data for the Cygnet and Tamar populations have been described in chapter 3. The total restriction endonuclease variation detected in the 58 second isochromosome lines from three Tasmanian populations is shown in figure 4.3.1. Overall, six of the eight enzymes (*Bam*HI, *Hin*dIII, *Hpa*I, *Pst*I, *Xho*I, and *Eco*RI) exhibited eight polymorphic restriction sites and three types of insertions/deletions (insertion 400bp, 280bp and deletion 200bp) were present in these lines. No variation was revealed by the eight restriction endonucleases in the *Adh* coding region or in the region of two putative loci (from -4kb to -5kb, and from 3'end of coding region to 1.1kb; see chapter 3). The haplotype variation in each population is listed in table 4.3.1, 4.3.2, and 4.3.3. The electrophoretic phenotypes, ADH-F and ADH-S, of the normal activity alleles are indicated.

In total, 11 polymorphic restriction endonuclease variants were found, although not all were present in each population. An analysis of the frequencies of the 11 restriction map variants in chromosomes bearing normal *Adh* alleles shows that the Cygnet and Huonville II samples do not differ from each other (X^2_{10} = 13.94, $p>0.05$) but they do differ from Tamar (X^2_{10} =18.69, $p<0.05$, and X^2_{10} =36.4, $p<0.001$, respectively. See figure 4.3.2)

The tabulations show that although 14 different haplotypes occurred each of the twelve *Adh* null-allele-bearing chromosomes had the same haplotype, regardless of the population from which they were derived or the year in which they were sampled. It is noteworthy that

the null haplotypes do not include either of the two insertions or the deletion detected in the Tasmanian populations.

Inspection of the data reveals that in the Tamar sample the three null alleles share the haplotype of one normal *Adh* allele, Tamar33. In the Cygnet samples two chromosomes, Cygnet19 and 25, and in the Huonville II population one chromosome, Huonville 16, have the haplotype of the null allele. Each of these four chromosomes carries the Adh^S allele.

The estimates of nucleotide-substitution variation and haplotype diversity in the chromosome bearing normal *Adh* alleles in the Tasmanian populations are shown in table 4.3.4. The calculations of estimates of the proportion of polymorphic nucleotides, p, overall heterozygosity, h, and estimated heterozygosity per nucleotide pair, θ, are the same as described in chapter 3. The levels of p, h and θ in the Cygnet and Tamar populations are similar to those previously reported in European and North American populations (Langley *et al.*, 1882; Birley, 1984; Cross and Birley, 1986; Aquadro *et al.*, 1986) and to those in Chinese and other Australia populations reported in chapter 3. However, the Huonville II population has significantly lower levels of p, h and θ, approximately half those of the other two Tasmanian populations. This may be related to a higher level of inbreeding in this population, which was collected in a dump of decaying apples.

Amongst the chromosomes carrying normal *Adh* alleles there were also some significant gametic disequilibria in each population (table 4.3.5). The same three disequilibria were present in Cygnet and Huonville II, *Hin*dIII(-3.0):D(0.2) and D(0.2):*Adh*, but none of them were significant in Tamar.

4.4 Discussion

Populations in south Tasmania (Cygnet and Huonville II) are separated from populations in the north of Tasmania (Tamar), as it is likely that there is limited migration between them. Between the northern and southern fruit growing regions of Tasmania there are few habitats suitable for *D. melanogaster* other than human habitats, and attempts to collect *Drosophila melanogaster* in the central region of the island were unsuccessful, although populations were expected to occur associated with human settlements (Gibson, J.B, personal communication). *Drosophila melanogaster* are abundant in Tasmania for only a few weeks in late April and early May.

The differences in the frequencies of restriction endonuclease variants and gametic disequilibria between Tasmanian populations of *Drosophila melanogaster* are unusual considering the small distances between the populations. Populations on the mainland , between which migration appears to be quite frequent, do not show differences in the frequencies of restriction endonuclease variants over such short distances. (Simmons *et al.*, 1989; and see chapter 3). The X^2 test for the differences in frequencies of restriction endonuclease variants between Australian populations (Table 4.4.1.a) indicated that there is no significant difference in frequencies of restriction endonuclease variants between all mainland populations except between Chateau Yarrinya and Coffs Harbour ($X^2_{14}=24.98$, $p < 0.05$) which were about 2000km apart from each other. All of the other significant differences occurred between populations involving Tasmanian populations. All three Tasmanian populations differ from a southern Australian population, Chateau Yarrinya.

The X^2 test for the analysis of the frequencies of restriction endonuclease variants between Chinese populations shows that most of

the populations differ from each other, the island population Haikou differs from all mainland populations. These data suggest that migration between populations on mainland China may be less extensive than on the Australian mainland, and that migration between mainland and island populations is limited in both countries.

A second main aim of the experiments described here was to investigate the molecular landscape surrounding the *Adh* locus in a sample of separately isolated null activity alleles so that they could be compared with one another and with normal alleles from the same populations to elucidate their origin. The results show that regardless of the population or year of origin, each of the 12 *Adh* null alleles studied has the same haplotype as revealed by eight hexanucleotide restriction endonucleases used in the experiments. The data for the Huonville II and Tamar populations are particularly revealing as eight and three null alleles respectively were available for comparison with normal alleles. Amongst the normal *Adh* alleles the null allele haplotype occurred at a frequency of 0.07 in each population yet, all three Tasmanian populations were identical ($p<0.001$), as were the eight null alleles from Huonville II ($p<0.001$).

It is significant that the null alleles do not contain any insertions or deletions in the size range which could be resolved with the technique used (greater than 50bp). This observation eliminates the possibility that the loss of ADH activity and the relatively high frequency of null alleles were brought about by the insertion of mobile elements of this size in or close to the *Adh* gene. In this context it is also worth mentioning that attempts to use *P* elements to induce mutations at the *Adh* locus have not been successful (Kidwell, 1986).

The data described here strongly suggest that the separately extracted null alleles from the Tasmanian populations share a common ancestry, which implies that the null alleles may well be copies of the same mutation. A possible, but in my view unlikely, alternative hypothesis which cannot be discounted with the present data, is that the null alleles derive from multiple mutations at an *Adh* allele in a specific molecular landscape. If this were so it might be expected that the separate mutations would possibly differ in structure and biochemical properties, but the previous work on *Adh* null alleles from Tasmanian populations mitigates against this hypothesis. Freeth *et al.*, (1987) found that all the nulls investigated here have been shown to produce mRNA's that are 100bp larger than normal and they each accumulate a precursor 1800bp RNA. The amount of the major transcript produced by the null allele is about 10% of that produced by normal alleles and none of the null alleles studied here had detectable levels of ADH cross-reacting material in western blot and enzyme-linked immunosorbent assay (ELISA) analyses (Freeth *et al.*, 1986).

The observation that the four chromosomes which share the null allele haplotype all carry the Adh^S allele suggests that the mutation which resulted in the loss of ADH activity occurred in an Adh^S allele. A further piece of evidence relevant to this argument is that all the Tasmanian null alleles bear the restriction site variant, *Bam*HI(-7.2), which is tightly linked to the Adh^S allele in most natural population of *Drosophila melanogaster* sampled world-wide (chapter3). Clearly, DNA sequence data from null alleles from the Tasmania populations are required to test fully this possibility, and to elucidate the molecular lesion leading to loss of ADH activity.

Gibson and Wilks (1989) cloned and sequenced (from base -64 to 1690 in the transcription unit, Kreitman, 1983) the *Adh* null allele,

Huonville II52. They compared the consensus sequence of this null allele with that of normal ADH activity alleles, and found that the nucleotide sequence of Huonville II52 contains eight extra bases in intron 2, adjacent to the 5′ splice site. The alteration in sequence appears to involve a ten base insertion and a two base deletion. The insertion includes an eight base pair duplication of an adjoining region. They suggest that this structural change alters transcription to give rise to an mRNA which is longer than normal and at 10% of the wild type level. They also found that twelve nucleotide substitutions and one insertion distinguished this null allele from the Adh^S consensus sequence. Except for one nucleotide difference, all of the other differences had been previously reported in various normal activity Adh^F alleles. Only two nucleotides in Huonville II52 are characteristic of the Adh^S rather than the Adh^F consensus sequence. At the sites responsible for the F/S polymorphism, the sequence of Huonville II52 is ACG, the same as Adh^F. These data suggest that the null allele Huonville II52 was in fact derived from an Adh^F allele, which is contrary to the suggestion made in this study. Recently Gibson and Wilks have also shown that other null alleles (from Cygnet and Tamar) have the same structure as Huonville II52 and thus the nulls from Tasmanian populations do have a common ancestry, as the present data suggests.

If, as seems likely, the null alleles turn out to be multiple copies of the same mutant, then the null has increased in frequency and spread to populations (at least 300km apart) which to some extent are genetically differentiated at the *Adh* locus. Tests of the Huonville II population show that null alleles with similar properties have persisted over at least three years and this population, in common with the other Tasmanian populations, experiences severe

bottle-necks in the winter months. There is little evidence, as yet, to suggest why the null allele increases in frequency. The estimates of the depression of heterozygote fitness, based on the average frequency of *Adh* null alleles in Tasmania, is 0.0003 (Freeth and Gibson, 1985). ADH activity is completely absent from the null homozygotes and the level in heterozygotes between the null allele and Adh^F or Adh^S is about half that of normal homozygotes (Freeth, Gibson and de Couet, 1986). This reduction is not trivial but in view of the data of Middleton and Kacser (1983) the difference may not be large enough to affect ethanol metabolism *per se*, although other reactions on which the enzyme impinges may be changed. There is evidence that heterozygotes between the nulls and normal alleles have a higher viability on ethanol media than expected from their ADH level (Freeth, Gibson and de Couet, 1986).

In view of the similarity of the Tasmanian null alleles it is interesting to analyse null alleles found in mainland populations to see whether they too share the same haplotype. A null allele was detected in the All Saint population in northern Victoria, but as this null was detected in a sample of frozen flies it could not be isolated. Another null allele was found amongst the chromosomes extracted from the Coffs Harbour population (the frequency is lower than 0.005) and which was maintained as a homozygous line. The restriction map of this variant carries an exceptional restriction site EcoRI(1.4), and it is different from that of the Tasmanian null alleles (see chapter 3). This site is not likely to be responsible for the loss of ADH activity. Gibson (unpublished data), using the PCR technique, amplified various regions of the *Adh* transcription unit in the variant and found that there is a 440bp deletion in exon 2, which is likely to be the cause of the loss of ADH activity.

These data indicate that *Adh* null alleles may arise from different mutations, but the high frequency and common origin of *Adh* null alleles in the Tasmanian populations suggests that the copies of this null allele may have a selective advantage in heterozygotes. Further studies on the fitness and population distribution of this null allele will be required to test this hypothesis.

Table 4.3.1. Restriction endonuclease map haplotypes in the Tamar population. In this and the following two tables the extracted chromosomes are ordered according to the ADH electrophoretic phenotype to aid comparisons

Line	*Bam* HI −7·2	Δ 280	*Hpa* I −6·9	*Hind* III −3·0	∇ 200	*Adh* +0·2	*Pst* I +1·1	*Xho* I +1·2	*Pst* I +1·3	Δ 400	*Bam* HI +3·9	*Eco* RI +9·0
Tamar												
1	−	−	+	−	−	*S*	−	+	+	+	+	−
17	−	−	+	−	−	*S*	−	+	−	−	+	−
19	+	−	−	−	−	*S*	−	−	−	−	−	+
33	+	−	−	−	−	*S*	−	+	−	−	−	+
50	+	−	−	−	−	*S*	−	+	−	−	−	−
12	−	−	−	−	−	*F*	−	+	−	−	−	−
29	−	+	+	−	−	*F*	−	+	−	−	−	−
30	−	−	−	−	−	*F*	−	+	−	−	−	+
32	−	−	−	−	−	*F*	−	+	−	−	−	−
40*	−	−	+	−	+	*F*	+	+	−	+	+	−
44	−	−	−	−	−	*F*	+	+	−	+	−	−
54	−	−	−	−	−	*F*	−	+	−	−	−	−
56	+	−	−	−	−	*F*	−	+	−	−	−	−
60	−	−	−	−	−	*F*	−	+	−	−	−	−
73	+	−	−	−	−	*F*	−	+	−	−	−	−
240	+	−	−	−	−	null	−	+	−	−	−	+
265	+	−	−	−	−	null	−	+	−	−	−	+
340	+	−	−	−	−	null	−	+	−	−	−	+

* The Tamar 40 chromosome has a complex structure, see **chapter 2.**

Table 4.3.2 Restriction endonuclease map haplotypes in the Cygnet population

Line	*Bam* HI −7·2	Δ 280	*Hpa* I −6·9	*Hin*d III −3·0	∇ 200	*Adh* +0·2	*Pst* I +1·1	*Xho* I +1·2	*Pst* I +1·3	Δ 400	*Bam* HI +3·9	*Eco* RI +9·0
Cygnet												
3	+	+	−	−	−	*S*	−	+	−	−	−	−
8	−	−	−	−	−	*S*	−	−	+	−	−	+
19	+	−	−	−	−	*S*	−	+	−	−	−	+
25	+	−	−	−	−	*S*	−	+	−	−	−	+
28	−	−	+	−	−	*S*	−	+	−	−	−	−
45	−	−	−	−	−	*S*	−	+	−	−	−	−
61	+	−	−	−	−	*S*	−	−	−	−	−	−
72	−	−	−	−	−	*S*	−	+	−	−	−	−
5	+	−	−	+	+	*F*	−	+	−	−	−	−
11	+	−	−	−	−	*F*	−	+	−	−	−	−
20	−	−	−	−	−	*F*	−	+	−	−	−	−
29	−	−	−	+	+	*F*	−	+	−	−	−	−
33	−	−	−	−	+	*F*	−	+	−	+	−	−
35	−	−	−	+	+	*F*	−	+	−	−	−	−
51	−	−	−	−	−	*F*	−	+	−	−	−	+
95	+	−	−	−	−	null	−	+	−	−	−	+

Table 4.3.3 Restriction endonuclease map haplotypes in the Huonville II population

Line	*Bam* HI −7·2	Δ 280	*Hpa* I −6·9	*Hind* III −3·0	∇ 200	*Adh* +0·2	*Pst* I +1·1	*Xho* I +1·2	*Pst* I +1·3	Δ 400	*Bam* HI +3·9	*Eco* R1 +9·0
Huonville II												
8	−	−	−	−	−	*S*	−	+	−	−	−	−
10	−	−	−	−	−	*S*	−	+	−	−	−	−
13	−	−	−	−	−	*S*	−	+	−	−	−	−
18	+	−	−	−	−	*S*	−	+	−	−	−	−
16	+	−	−	−	−	*S*	−	+	−	−	−	+
14	−	−	−	−	−	*S*	−	+	−	−	−	−
3	−	−	−	−	+	*F*	−	+	−	−	−	+
4	−	−	−	−	−	*F*	−	+	−	−	−	−
5	−	−	−	+	+	*F*	−	+	−	−	−	−
6	−	−	−	+	+	*F*	−	+	−	−	−	−
7	−	−	−	−	−	*F*	−	+	−	−	−	−
9	−	−	−	+	+	*F*	−	+	−	−	−	−
12	−	−	−	−	−	*F*	−	+	−	−	−	+
15	−	−	−	+	+	*F*	−	+	−	−	−	−
17	−	−	−	−	−	*F*	−	+	−	−	−	+
19	−	−	−	+	+	*F*	−	+	−	−	−	−
98	+	−	−	−	−	null	−	+	−	−	−	+
70	+	−	−	−	−	null	−	+	−	−	−	+
144	+	−	−	−	−	null	−	+	−	−	−	+
9	+	−	−	−	−	null	−	+	−	−	−	+
77*	+	−	−	−	−	null	−	+	−	−	−	+
52†	+	−	−	−	−	null	−	+	−	−	−	+
36‡	+	−	−	−	−	null	−	+	−	−	−	+
41†‡	+	−	−	−	−	null	−	+	−	−	−	+

* Homozygous lethal chromosome

† Homozygotes sterile

‡ Extracted in 1984 whereas all other chromosomes in this population were extracted in 1985

Table 4.3.4 Estimates of the proportion of polymorphic nucleotides, p, overall heterozygosity, h (Nei and Tajima, 1981) and estimated heterozygosity per nucleotide pair, θ (Ewens, Spielman and Harris, 1981). Standard errors are given in parentheses and that for $\hat{\theta}$ assumes free recombination

Population	p	h	θ
Tamar	0·019 (0·007)	0·86 (0·057)	0·007 (0·0026)
Cygnet	0·017 (0·007)	0·95 (0·014)	0·006 (0·0024)
Huonville II	0·009 (0·004)	0·85 (0·06)	0·003 (0·0015)

Table 4.3.5 Significant gametic disequilibria in the Tasmanian populations

Population	Number of comparisons	Significant gametic disequilibria
Tamar	55	*Bam* HI (−7·2): *Adh*, $p < 0·01$ *Hpa* I (−6·9): *Bam* HI (+3·9), $p < 0·01$ *Adh* (+0·2): *Bam* HI (+3·9), $p < 0·01$ *Pst* I (+1·1): Δ 400, $p < 0·01$ *Xho* I (+1·2): *Eco* RI (+9·0), $p < 0·05$
Cygnet	45	*Hind* III (−3·0): *Adh*, $p < 0·05$ *Hind* III (−3·0): ∇ 200, $p < 0·001$ ∇ 200: *Adh* (+0·2), $p < 0·05$
Huonville II	10	*Hind* III (−3·0): *Adh*, $p < 0·05$ *Hind* III (−3·0): ∇ 200, $p < 0·001$ ∇ 200: *Adh* (+0·2), $p < 0·05$

Table 4.4.1. a. X^2 test for the analysis of the frequencies of the restriction endonuclease variants between the eight Australian populations. Degrees of freedom are given in the parentheses.

populations	Cygnet	Tamar	Chateau Yarrinya	All Saints	Araluen	Coffs Harbour	Cardwell
Huonville	13.94(10)	36.4** (10)	25.8** (10)	23.69** (12)	12.87(9)	19.85* (10)	20.39(12)
Cygnet	-	18.07* (10)	23.05* (12)	17.54(14)	7.4(11)	13.19(11)	12.18(14)
Tamar	-	-	40.13*** (14)	14.57(13)	11.99(12)	15.28(11)	17.07(13)
Chateau Yarrinya	-	-	-	15.56(13)	20.20(12)	24.98* (14)	17.04(15)
All saints	-	-	-	-	10.52(13)	20.45(14)	19.62(16)
Araluen	-	-	-	-	-	13.04(12)	14.98(14)
Coffs Harbour	-	-	-	-	-	-	11.02(12)

* $p < 0.05$, ** $p < 0.01$.

Table 4.4.1.b X^2 test for the analysis of the frequencies of the restriction endonuclease variants between six Chinese populations.

populations	Jinan	Shanghai	Fuzhou	Guangzhou	Haikou
Dalian	19.63* (9)	19.64* (10)	10.79 (7)	11.40 (7)	22.89* (11)
Jinan	-	24.75** (10)	12.80* (6)	34.34** (14)	38.07*** (12)
Shanghai	-	-	22.06** (9)	26.7** (10)	40.04*** (15)
Fuzhou	-	-	-	11.49 (6)	26.41** (11)
Guangzhou	-	-	-	-	20.88* (11)

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 4.3.1 Restriction endonuclease variations detected in the *Adh* region of three Tasmanian populations. 3′ is to the right of the figure. The boundaries of the *Adh* transcription unit are indicated and the 5′ leader sequence and three exons are shaded. Variable restriction endonuclease sites are indicated below the map; triangles represent insertions (points upward) or deletions (points downward) and the estimated sizes are shown.

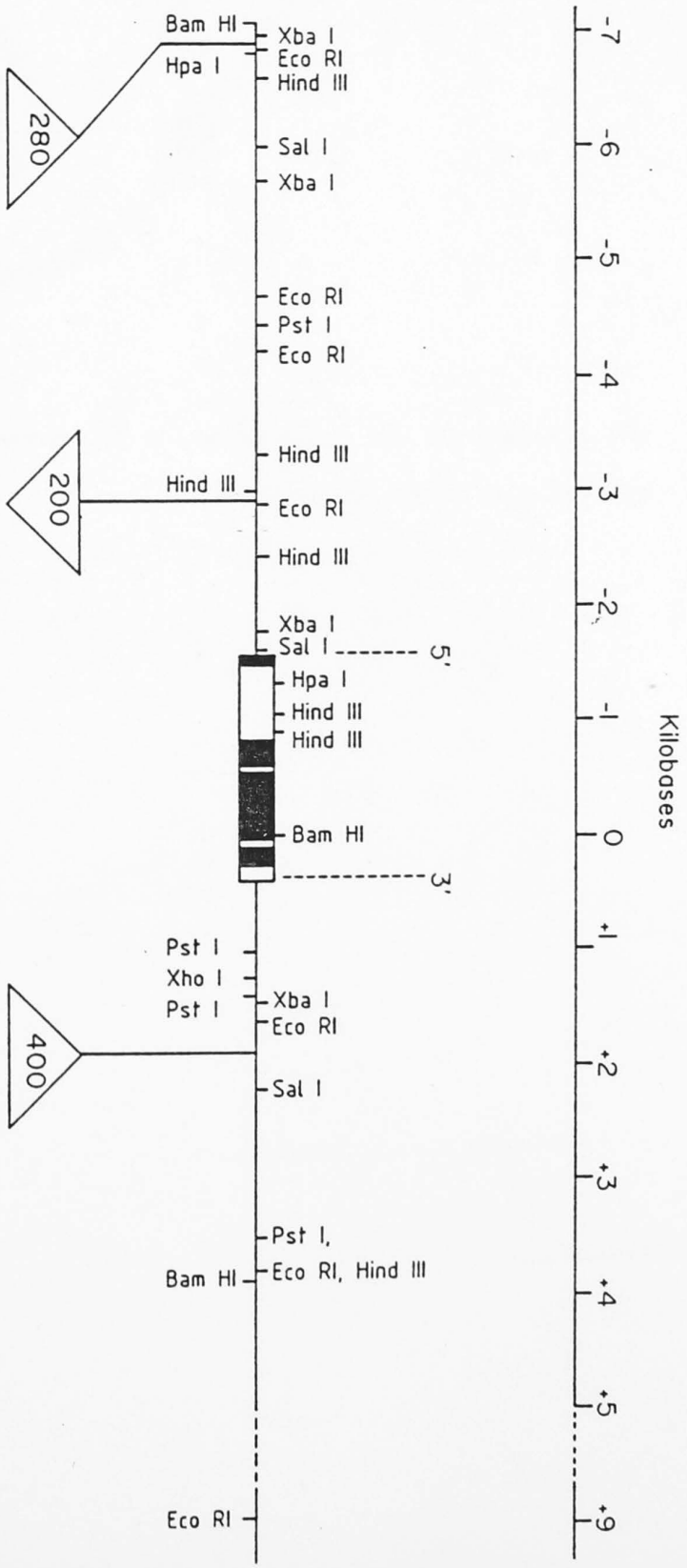
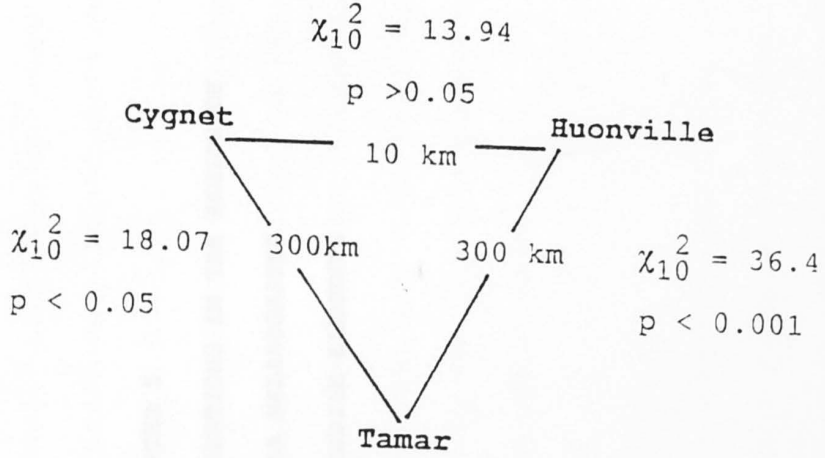

Bam HI
Xba I
Eco RI
Hpa I
Hind III
280
Sal I
Xba I
Eco RI
Pst I
Eco RI
Hind III
200
Hind III
Eco RI
Hind III
Xba I
Sal I
5'
Hpa I
Hind III
Hind III
Bam HI
3'
Pst I
Xho I
Xba I
Pst I
Eco RI
400
Sal I
Pst I,
Bam HI
Eco RI, Hind III
Eco RI
-7
-6
-5
-4
-3
-2
-1
0
+1
+2
+3
+4
+5
+9
Kilobases

Figure 4.3.2 X^2 test for the analysis of the frequencies of the eleven restriction map variants between the Tasmanian populations.


$\chi^2_{10} = 13.94$
p >0.05
Cygnet
Huonville
10 km
$\chi^2_{10} = 18.07$
p < 0.05
300km
300 km
$\chi^2_{10} = 36.4$
p < 0.001
Tamar

CHAPTER 5

IDENTIFICATION OF INSERTIONS IN THE REGION OF *Adh* GENE OF *DROSOPHILA MELANOGASTER* WITH KNOWN MOBILE ELEMENTS

Chapter 5. Identification of insertions in the region of *Adh* gene of *Drosophila melanogaster* with known mobile elements.

5.1 Introduction

The study of restriction map variation in the *Adh* region of *Drosophila melanogaster* in the Australian and Chinese populations revealed ten types of insertions, which vary in size and positions (chapter 3). These insertions may represent part of the DNA sequence of mobile elements. Mobile elements play important roles in gene mutation, expression, and regulation in prokaryotes and eukaryotes (Shapiro,1983).

Mobile elements are moderately repetitive sequences dispersed in the genome. The number of copies, and the positions of the elements differ between stocks. About 12% of the *Drosophila melanogaster* genome (about 20000kb) consists of moderately repetitive DNA (Young, 1979). At least four distinct structural classes of mobile elements have been identified in the *Drosophila melanogaster* genome:

1.*Copia*-like elements (Rubin, 1983)

In total, there are over 30 different families of *copia*-like elements, accounting for about half of the moderately repetitive DNA in the *Drosophila* genome. More than nine such repeat-sequence families, with element sizes ranging from 5 (*copia*) to 9.3kb (*roo*), have been studied in detail. Elements from each family carry long direct terminal repeats (271-571bp), each repeat being about 5% of the length of the element. The integration and excision of the elements are site-specific. Transposition of an element to a new site results in the duplication of a short sequence that pre-existed at the integration site, generating a short direct repeat of host sequence

(4-12bp) flanking the inserted element. *Copia*-like elements are present at about 10-100 copies, and are located at widely scattered sites in the chromosome arms and in the centric heterochromatin of *Drosophila melanogaster*. In general, the copies from one family are quite similar to one another in sequence. Usually, *copia* elements are transcribed, producing abundant RNA.

2.*Foldback (FB)* elements (Potter *et al.*, 1980; Truett *et al.*,1981)

The members of this family (e.g. *FB1, FB2, FB3, FB4, FB5,* w^c, w^{DZL} *and TE* etc.) have long terminal inverted repeats which are homologous in all family members. However, the length of the inverted repeat and the non-repeat central region are highly variable. The inverted repeat sequences themselves are internally repetitious, having a sub-structure made up primarily of 31bp tandem repeats. The *FB* elements disperse throughout the genome with about 30 sites on all chromosomes and exhibit polymorphism in their genomic positions. The transpositions of *FB* elements create duplications of 2-11bp sequences at the new insertion sites. This short 2-11bp stretch initially presents only once at the target site.

3.*P*-elements (Kidwell *et al.*, 1977; Rubin, 1983)

P-elements,with structures distinct from those of the *copia*-like or *FB* element, play a central role in hybrid dysgenesis. They carry perfect inverted repeats of 31bp. A fraction of the *P* elements are very similar in sequence to one another and are 2.9kb in length. The reminder of the *P* elements are more heterogenous, but all appear to have structures that are consistent with their having been derived from the 2.9kb element by one or more deletions. Upon transposition an

8bp sequence at the insertion site is duplicated. In *P and Q* strains there are usually 30-50 *P*-element copies per haploid genome.

Previous work on the distribution of *P* elements showed a gradually decreasing frequency of *P* element from western Europe, where most strains are *Q* type (carry a subset of the *P* element family that lacks sterility potential), to middle Asia, where *M* type strains (which do not show activity of *P* element nearly all of them carry defective *P* elements, which are called KP and repress transposition of complete *P* elements) predominate (Anxolabehere *et al.*, 1985). The frequency of *P* element decreases from Far East (Japan and east coast of China) to middle Asia (David and Capy, 1982). In *Drosophila melanogaster* populations from the east coast of Australia the frequency of *P* elements decreases from north to south (Boussy and Kidwell, 1987). This overall pattern is hardly consistent with selective factors acting on the frequency of *P* element since the geographic differentiation is in different directions. These patterns have been explained by the recent invasion of *P* element (David and Capy 1988). The *P* element was first found in American populations after 1940, and the flies collected before 1940 are all *M* type and are devoid of any *P* element. During the period 1940-1960 *P* elements invaded populations all over the world. In the process the loss of some or all of the original *P* functions may have occurred, giving rise to *Q* strains (Kidwell, 1983). Boussy *et al.*, (1988) found a *KP* element cline in populations from eastern Australia which is reciprocal to that of the full-size *P* elements: the frequency of *KP* is low in populations in the north, and increases towards the south, *M* type flies from south of Australia, Tasmanian flies actually possess the highest number of *KP* elements. Compared with full-size *P* elements the *KP* element lost some

functional fragments (Rubin, 1983), which may account for the lack of active *P* elements and male recombination in Tasmanian populations.

4.*F* elements (Dawid *et al.*, 1981).

These DNA segments are homologous to type 1 ribosomal DNA insertions which interrupt about 50% of rDNA repeats on the X chromosome and were called insertion-like elements (Wellauer *et al.*, 1978; Dawid *et al.*, 1981). The *F* element interrupts a ribosomal insertion-like element. When *F* elements insert into a ribosomal insertion-like element, 13bp at the target site of the insertion-like element are duplicated. Within the *F* element no extended direct or inverted repeat sequence exists. The *F* elements are transcribed into rare, heterogeneous, poly(A)-lacking nuclear RNA molecules (Dawid *et al.*, 1981).

5.Other mobile elements

A number of other mobile elements have been found in the genome of *Drosophila melanogaster*, including the *Hobo* element, which is similar to the *P* element (McGinnis *et al.*, 1983), and the I factors, which control *I-R* hybrid dysgenesis (Bucheton *et al.*, 1976).

The first evidence for mobile elements in *Drosophila melanogaster* was provided by the behavior of a small number of unstable mutations which revert to a wild-type at an unusually high rate and generate deletions and other chromosomal rearrangements having one endpoint at the site of the mutation (Green, 1976, 1977a, 1980; Golubovsky *et al.*, 1977). These mutations are the result of DNA insertions. Good examples of the function of mobile elements are the mutations at the white locus of *Drosophila melanogaster* (Rubin, 1983). The *white-crimson* (w^c) mutant arose by the insertion of a 10kb *FB* element into the *white*

ivory allele. The *white dominant zeste-like* (w^{DZL}) mutant resulted from a 13kb insertion consisting of two *FB* elements flanking a segment of nonrepetitive DNA. The *white-apricot and* $w^{hb81b11}$ mutants were caused by the insertion of a *copia* element in different positions. The mutants $w^{\#6}$, $w^{\#12}$, $w^{hd80k17}$ *and* w^{h381b9} were caused by insertions of members of the *P* element sequence family. Also mutations w^{IR1} *and* w^{IR3} are associated with a 5.4kb *I* factor insertion within the *White* locus.

Aquadro *et al.*, (1986) in surveying restriction endonuclease variation in the *Adh* gene region found that apart from the small insertions, 7 types of big insertions in the 12kb *Adh* gene region which range from 0.34kb to 10.2kb were homologous to one or more mobile elements: insertions 0.34kb, 0.4kb, 0.7kb and 10.2kb, which were located in the 0.23kb *EcoRI/SalI* fragment 1.5kb 3′ to *Adh*, showed homology to *cDM2161* which is similar to an *F* element in structure. Also the 10.2kb insertion and 0.34kb insertion showed homology to *B104*. A 4.8kb insertion showed homology to *F101* element; a 5.2kb insertion showed homology to a *copia* element and was associated with low ADH activity. (refer to Figure.3.4.1)

Some of the big insertions occurring in the *G6pd* gene region of *Drosophila melanogaster* were identified (Earnes *et al.*, 1989): an *F* element bearing pDM101 clone hybridised *in situ* at the site of 5kb insertions in the intron 1 in each of the three lines; and a 10kb insertion in intron 1 hybridised to *B104 in situ*.

In the work described in this chapter, ten types of insertions found in the Australia and Chinese populations were cloned in an attempt to find out whether any of the insertions were homologous to known mobile elements, whether the type, frequencies and distribution of mobile elements in Australian and Chinese populations are similar to those in other populations.

5.2 Materials and Methods

5.2.1 Clones for mobile elements

The clones of the mobile elements were kindly provided by Dr.G. Miklos, Dr.J. Oakeshott. and Dr. I.B. Dawid.

pπ 25.1 is a *Bam*HI fragment containing a complete 2.9kb P element cloned into the pBR322 vector (O'Hare and Rubin, 1983).

Copia is a *Bam*HI fragment containing a 5.0kb *copia* element cloned in pBR322 (Rubin *et al.*, 1976).

B104B is an *Eco*RI fragment containing a 2.8kb *B104B* which is part of the *B104* element, cloned in *PACYC184* vector (Scherer *et al.*, 1982).

pI407 is a *Sal*I fragment containing a 5.4kb *I* factor cloned in the *PAT153* vector (Bucheton *et al.*, 1983).

cDm412 is a *Hin*dIII fragment containing a 7kb 412 *copia*-like element cloned in pBR322 (Rubin, *et al.*, 1981.)

pPW297 is a *Hin*dIII fragment containing a 6.5kb 297 *copia*-like element cloned in pBR322 (Finnegan *et al.*, 1978; Rubin *et al.*, 1981).

pDmI-101 is a *Eco*RI fragments containing a 4.3kb *F* element cloned into pBR322 (Dawid *et al.*, 1981 and Dawid, I.B. personal communication).

5.2.2 Extraction of plasmid DNA

This was carried out as described in chapter 3.

5.2.3 Cloning the insertions

1.Preparation of insertion DNA from single second chromosome lines

Genomic DNA from second isochromosome lines which had been shown to contain an insertion was extracted by Chia's method (Chia *et al.*, 1985, see chapter 4). The concentration of DNA was estimated with 0.4%

agarose mini-gel electrophoresis in TBE buffer (90mM Tris, 90mM Boric acid, 10mM EDTA pH=8) with ethidium bromide (0.15µg/ml). Using a series of lambda DNA dilutions as controls, a series of DNA dilutions were run on the mini-gel at 68V for 1.5 hours. Preliminary experiments using Southern blots revealed *Bgl*II sites at each of the two ends of the 12kb sAF2 fragment. All of the insertions are included in the 11.8kb *Bgl*II fragment. Genomic DNA was digested completely by the restriction enzyme *Bgl*II at 37°C for at least 4 hours or overnight under the conditions recommended by the manufacturer (Amersham). After digestion part of the sample was run on a 1% agarose gel, then transferred to a nitrocellulose membrane for Southern blotting (described in chapter 3) using sAF2 DNA as a probe to check that there was no other *Bgl*II site in any of the insertions.

The remaining DNA sample was extracted with the same volume of phenol-chloroform (1:1), then centrifuged at 12,000rpm for one minute. The supernatant was transferred to a fresh Eppendorf tube, 0.1 volume of 3M NaCl was added, then 2 volumes of cold ethanol, and mixed by inversion. The tube was left at -90°C overnight, and then centrifuged at 12,000rpm for ten minutes. The white DNA pellet was washed with 70% ethanol, dried in vacuum for seven minutes, and then resuspended in TE solution. The amount of DNA lost in the procedure was about 30%.

2.Ligation and packaging

EMBL-3 lambda-phage was chosen as the vector for the cloning (Frischauf *et al.*, 1983). This vector has a polylinker cloning site, *Sal*I-*Bam*HI-*Eco*RI. Its cohesive ends are complementary to those produced by *Bgl*II. This vector allows insertion of DNA ranging in size between 8 and 23kb, which covers the length range of all the *Bgl*II fragments containing the insertions, which were found in this study.

1µg EMBL-3 arms (Amersham), 0.5µg insertion DNA, 1µl 10X ligation buffer (10mM Tris-HCl pH7.5, 10mM $MgCl_2$), 1µl 100mM DTT, 1µl 10mM ATP, 1µl 100µg/ml BSA and 1 unit T4 ligase were made up to the volume of 10µl, mixed by tip, and left at room temperature for three hours. The mixture was added to Amersham's Extract A (10µl aliquots of an extract prepared from *E.coli* strain BHB2688) followed by adding 15µl Amersham's Extract B (extract prepared from *E.coli* strain BHB2690), tipped and centrifuged for ten seconds, then left at room temperature for two hours. 0.5ml phage dilution buffer (20mM Tris-HCl pH7.4, 100mM NaCl, 10mM MgCl) was added to the package, then 25µl chloroform was added, mixed well by invertion, and then stored at 4°C.

3.Screening and plaque purification of the insertion clones

Using *E.coli* LE392 strain as host, the recombinant phages were plated on NZCYM+M media (10g NZ amine, 5g NaCl, 1g casamino acid, 5g Bacto-yeast-extract, 2g MgCl and 2g maltose per liter, pH 7.5 with NaOH.). At least six plates (containing about 6000 clones) for each package were screened by plaque hybridisation using the sAC1 clone (which contains a 4.7kb *EcoRI* fragment from position -2.6kb to 2.1kb, Goldberg, 1980) labelled with α^{32}PdCTP by nick-translation (as described in chapter 3) as a probe. The DNA from plaques on the plates were transferred to marked circular nitrocellulose membranes by blotting for ten minutes. The membranes were soaked in Blot I solution for 5 minutes, in Blot II for 5 minutes and in 2XSSC for 10 minutes (solutions were the same as described in chapter 3), then dried by blotting twice between two sheets of blotting paper and baked in a 80°C vacuum oven for three hours. The membranes were hybridized with sAC1 probe (as described in chapter 3) to detect positive clones. The positive clones were picked and put in 1ml SM buffer in an Eppendorf

tube, and 25µl chloroform was added. A series of dilutions of this phage solution were re-plated and the plaques were screened. Positive plaques were picked which were quite isolated in the plate (containing about 20-30 plaques). The procedure was repeated 3-4 times until the positive clone was purified (Benton and Davis, 1977).

4.Hybridisation

The purified plaques were plated and the DNA from each type of insertion was transferred to two circular nitrocellulose membranes (two lifts). The membranes were cut into four pieces which were marked and each piece was probed by one P^{32} labelled mobile element. sAC1 probe was used as control

5.3 Results

Ten different insertions (insertion 0.28, 0.35, 0.4, 0.48, 0.7. 1.0. 1.5, 3.0, 4.5 and 5.0kb) were cloned. The results of the hybridizations are shown in table 4.3. Two of the cloned insertions showed homology in DNA sequence to known mobile elements. Insertion 0.28 showed homology to *B104B*, which usually has 100 copies in the genome of *Drosophila melanogaster*. This type of insertion occurs in the Cygnet (C3), Tamar (T29) and All Saints (A11) populations (see chapter 3). Another insertion, insertion 0.4kb, showed homology in DNA sequence to mobile element *F101*, which usually has 25 copies in the *Drosophila melanogaster* genome. Insertion 0.4kb occurred in all Australian and Chinese populations with a frequency ranging from 0.07 (Tamar) to 0.47 (Haikou).

5.4 Discussion

Because of the limited number of mobile elements available to me to test, most of the insertions detected in my study have not been identified, especially for the cluster of insertions in the 0.23kb *EcoRI/SalI* fragment. The 0.28kb insertion found to be homologous to a *Copia-like* element, *B104B*, occurred only in the Tasmanian and southern Australian populations. It was not found in the Chinese and other Australian populations. This insertion is much shorter in length than *B104B* (*B104B* is a 2.8 kb *XbaI/AvaI* fragment in the 8.7kb *B104* region), and it could be part of the mobile element. A typical *B104* element is 8.7kb in size and flanked by direct terminal repeats 429bp long. This element accounts for 0.4% of the *Drosophila melanogaster* genome, and has most abundant copies compared with other mobile elements and produces abundant poly(A)$^+$-RNA in early embryos (Scherer *et al.*, 1981, 1982). The insertion of a *B104* element causes a 5 base pair repetition. There are various small insertions and deletions in different *B104* elements. Scherer *et al.*, (1982) found a clone, B102, which was 1kb shorter than B104; Finnegan *et al.*, (1978) found that a 2.4kb fragment inserted in *B104* was part of a cDM412 clone. Wensink *et al.*, (1979) found that *Drosophila melanogaster* DNA contains clusters of short repetitive sequences (less than 1kb) scattered over a large number (1000) of chromosome regions, and some of the clusters hybridise to the region of the cDM412 clone which contains the *B104* fragment with the 2.4kb insertion. The insertions 0.28kb and 0.4kb may share the characteristics of the above short repetitive sequences.

The insertion 0.4kb found in the Australian and Chinese populations looks similar to that from the North American populations in size and location (See figure 3.3.1 and Aquadro *et al.*, 1986). In my study the insertion 0.4kb was homologous to *F101* whilst the

insertion 0.4kb found in North American populations was homologous to *cDM2161* which is an *F*-like element (Aquadro *et al.*, 1986). However the two elements are similar in structure.

None of the insertions in the *Adh* gene region found in present study were homologous in sequence to *P* elements and this is consistent with the results of Aquadro *et al.*, (1986). Also none of the insertions in the Australian and Chinese populations were found homologous to *Copia* elements, this kind of insertion ~~were~~ was found in one line in the study of Aquadro *et al.*, (1986) and the insertion reduced the ADH activity of this line. Most of the insertions found in my study have little effects on the expression of *Adh* gene, except a 5.0kb insertion in intron I, but this insertion was not homologous to the mobile elements tested here.

The insertion or excision of mobile elements induces gene mutation. Green, (1977) and Golubovsky *et al.* (1977) observed that some second chromosomes of *Drosophila melanogaster* from wild caught flies increase the frequency of mitotic crossing over. Such second chromosomes were designated MR (male recombination) chromosomes, and a subset of *P* elements on the second chromosome induces male recombination (Kidwell, 1986). When MR chromosomes are introduced into laboratory stocks inordinately high rates of unstable, putative insertion mutations at the X-linked *yellow (y)*, *raspberry (ras)* and *singed (sn)* loci are observed. The excision of *P* elements is usually precise (±50bp, Rubin, 1982) since MR-induced mutants revert to stable wild type, however not all excision is precise and mutable genes generate deletions (Green, 1980). Tschudi *et al.*, (1982) observed that the insertion or excision of the *B104* element induced a rearrangement of the *5S* RNA gene. Similarly, the insertion or excision (precise or not precise) of other *Copia*-like, *I* and *FB* elements also induces gene

mutation. Whether the insertions homologous to mobile elements in the *Adh* region are transposible or not, is not clear yet; however, they seem to have no effects on the action of the gene.

Table 5.3 cloned insertion lines and the results of their hybridisation with known mobile elements.

+ denotes positive hybridisation, - denotes negtive hybridisation.

line	insertion	mobile element probes						
	size(kb)	Copia	B104B	412	297	PI 407	F101	Pπ25.1
T29	o.28	-	+	-	-	-	-	-
H2	0.35	-	-	-	-	-	-	-
C.Y.1	0.4	-	-	-	-	-	+	-
Sh39	0.48	-	-	-	-	-	-	-
CD5	0.7	-	-	-	-	-	-	-
C.Y.1	1.0	-	-	-	-	-	-	-
H4	1.5	-	-	-	-	-	-	-
Ar13	3.0	-	-	-	-	-	-	-
Cd15	4.5	-	-	-	-	-	-	-
Al13	5.0	-	-	-	-	-	-	-

CHAPTER 6

VARIATION IN ADH ACTIVITY IN AUSTRALIAN AND CHINESE POPULATIONS OF *DROSOPHILA MELANOGASTER*

Chapter 6 Variation in ADH activity in Australian and Chinese populations of *Drosophila melanogaster*.

6.1 Introduction

The activity of the *Adh* gene in *Drosophila melanogaster* may be an important factor in the mechanism maintaining the *Adh* variation in natural populations. In *Drosophila melanogaster* there is single *Adh* locus located on the second chromosome at map position 50.1 (Grell *et al.*,1965) within the polytene chromosome bands 35B1-5, most probably within 35B2 (O'Donnel *et al.*,1977; Woodruff and Ashburner, 1979a,b). The *Adh* gene in *Drosophila melanogaster* is polymorphic in most natural populations with two common electrophoretically detectable alleles. Electrophoretic variation at the *Adh* locus was first reported by Johnson and Denniston (1964). Generally there are two common electrophoretic alleles in most natural populations: the Adh^F (F) and Adh^S (S) alleles. The enzymes encoded by Adh^F and Adh^S alleles differ by a single amino acid at residue 192, with lysine in ADH-S replaced by threonine in ADH-F (Fletcher *et al.*, 1978; Thatcher, 1980). A thermostable allele, Adh^{FChD}, is found at low frequency at some populations, (Milkman, 1976, Sampsell, 1977; Lewis and Gibson, 1978; Wilks *et al.*, 1980) and differs from Adh^F by the substitution of serine for proline at residue 214. The *Adh* gene produces abundant protein, nearly 1% of total soluble protein in the adult fly (Ursprung *et al.*, 1970).

The structure of *Adh* gene is shown in figure 6.1. The protein encoding sequence consists of three exons (of lengths 96bp, 405bp and 264bp) separated by two small introns (65bp and 70 bp). Two transcripts are produced, a larval and an adult form, differing only in their 5' non-coding leader sequences. The gene is transcribed from

two different promoters at different life stages (Benyajati *et al.*, 1983); one promoter (adult or distal) is active transiently in embryos, at a moderate level in third instar larvae, and at a high level in adults. The other promoter (larval or proximal) is used principally in late embryos and all larval stages, and at low level in adults.

The ADH (EC 1.1.1.1) of *Drosophila melanogaster* contains two identical subunits, each 254 residues in length and of molecular weight 27,400. The function of this enzyme is unclear except that it is implicated in the detoxification of environment alcohols. ADH catalyzes the oxidation of alcohols to aldehydes or ketones and concurrently reduces NAD^+ to NADH (Grell *et al.*,1968).The complete amino acid sequence for the wild type ADH protein has been determined (Sofer and Ursprung, 1968).

ADH activity in *Drosophila melanogaster* varies between different parts of the body (Ursprung *et al.*,1970; Korotchkin *et al.*,1972), between sexes, and between life stages (Dunn *et al.*, 1969; Ursprung *et al.*, 1970; McDonald and Avise, 1976). In addition the level of ADH activity is affected by different environmental and nutritional conditions (Clarke *et al.*, 1979; Gibson and Wilks, 1987). ADH activity also varies between the electrophoretic *Adh* phenotypes (Rasmuson *et al.*, 1966; Gibson, 1970; Vigue and Johson,1973; Day *et al.*, 1974a;) and between strains of the same *Adh* genotype (Gibson and Miklovich, 1971; Ward and Hebert, 1972; Birley and Barnes, 1973, 1975;). In general, larvae and adults from homozygous Adh^F strains possess greater in vitro ADH activity than those from Adh^S strains and Adh^{FChD} strains. (Gibson, 1972; Lewis and Gibson, 1978; Maroni, 1978; McDonald *et al.*, 1980; Anderson and Gibson, 1985; Aquadro *et al.*, 1986). Adh^{FChD} possesses intermediate ADH activity, with a substrate

specificity like ADH-S, and an electrophoretic mobility like ADH-F. Lewis and Gibson (1978) investigated the level of ADH activity and ADH protein in eight ADH-F isofemale lines, seven ADH-S and one ADH-FChD isofemale lines, they found that the mean level of ADH activity of ADH-F lines was nearly twice as high as that of ADH-S lines in the third instar larval stage, and ADH-F lines possessed more ADH protein than ADH-S lines. Enzyme extracted from ADH-F lines had a higher mean catalytic efficiency per enzyme molecule than that from ADH-S lines. They also found that the ADH-FChD line had intermediate levels of ADH activity and ADH protein.

Anderson and Gibson (1985) investigated ADH activity in seven populations sampled from the island of Tasmania and south-east Australia (range covered latitudes from 34.3°S to 43.2°S). They found that between and within population variation in ADH activity and ADH protein in flies in the wild is mainly due to the electrophoretic phenotype, ADH-F or ADH-S. Aquadro *et al.* (1986), investigated 48 lines from four east United States populations, and found that ADH activity was spread over a five fold range, with the mean ADH activity of Adh^F lines about twice high as that of Adh^S lines.

Linked and unlinked modifier loci have also been shown to affect ADH activity in natural population of *Drosophila melanogaster* (Ward and Hebert 1972; Thompson *et al.*, 1977; Mcdonald and Ayala, 1978; Birley *et al.*,1980; Laurie-Ahlberg *et al.*, 1980; Maroni *et al.*, 1982). Maroni and Laurie-Ahlberg (1983) found a variety of modifier loci in three Adh^F and three Adh^S lines extracted from a natural population. The differences in ADH activity levels among Adh^F strains were due to multiple modifier loci with trans-acting effects, most of which were located on the second chromosome. The differences in ADH activity levels between different Adh^S strains were found to be due to modifier

sites located very close to the structural gene. The modifiers found in Adh^S strains were *cis*-dominant. It has been shown that the modifiers of ADH activity are located on the X, second and third chromosomes(Pipkin and Hewitt, 1972a; Hewitt *et al.*, 1974; Barnes and Birley, 1975, 1978). Birley *et al.*(1981) located a modifier on the third chromosome, which affected ADH activity level without apparently changing the amount of ADH protein. The modifiers could influence ADH activity by changing the rate of mRNA processing or translation. They might also affect the stability of the mRNA or the protein.

Study of the expression of the *Adh* gene has been carried out by introducing fragments of the region of the gene into the germ line of *Adh* null flies (Goldberg *et al.*,1983). These studies indicated that all of the *cis*-acting DNA sequences required for normal *Adh* gene expression *in vivo* are contained within an 11.8kb *SacI* restriction fragment which includes 5.5kb and 4.5kb of 5′ and 3′ sequences flanking the *Adh* locus. In further studies (Posakony *et al.*, 1985) deletions were introduced into the cloned *Drosophila melanogaster Adh* gene and the effects of the mutations on *Adh* gene expression *in vivo* were observed. These authors proposed that the expression of the *Adh* gene in *Drosophila melanogaster* is regulated by *cis*-acting sequence elements in at least three different regions both upstream of and within the transcription unit. One region lies over 2kb upstream from the proximal promoter, and is necessary for normal levels of *Adh* transcription in larvae. A second region is located between -69 and -660bp from the distal transcription start point, and contains elements that confer temporal and tissue specificities characteristic of the distal promoter. The third region is located 3′ to position -386bp from the proximal transcription start point, and is responsible for the tissue specific activity of the proximal promoter in larvae.

In studies of inbred lines the survivorship of Adh^F on alcohol containing medium has generally been found to be higher than that of Adh^S (Oakeshott, 1976; Kamping and van Delden, 1978). These results suggested ethanol could be a significant selective factor in laboratory population. Alcohol is the substrate for ADH, and is of special ecological importance. *Drosophila melanogaster* has high alcohol tolerance compared with most other species of *Drosophila melanogaster* group (David *et al.*, 1979). The alcohol tolerance seems to be associated with the concentration of alcohol in the environment. Gibson and Wilks (1988) investigated the ethanol tolerance of *Drosophila melanogaster* populations in 26 breeding sites in an orchard, grape skin dumps, open fermentation vats and a winery, where the average ethanol level were different, from low to high. The results show significant difference in ethanol tolerance between habitats. The proportion of surviving flies, which were kept on standard media with 9% ethanol for six days, were 23.2%, 24.7%, 42.1% and 54.4 (from low level of alcohol to high level of alcohol) for males, and 33.2%, 35%, 36,8% and 47.3% for females respectively. In temperate regions, huge populations of *Drosophila melanogaster* build up in wineries in the autumn. The flies breed in the habitats with a high level of alcohol, which may contain more than 10% ethanol. In the tropics *Drosophila melanogaster* breeds mainly in fruit, with a lower level of alcohol (Lemeunier *et al.*, 1986). David and Bocquet (1975b) reported that between tropical Africa and Europe there is latitudinal cline in alcohol tolerance. Adult tolerance, measured by the concentration killing 50% of adults after two days exposure (LC 50), increases from 6 to 16% ethanol between the equator and Europe. Also Parsons (1980a) reported an alcohol tolerance cline in Australian populations. Samples were collected along the Australian coast at

sites at Melbourne, Brisbane, Townsville, Darwin and Melville Island. The tolerance was measured by the number of hours at which 50% flies had died (LT 50) in the vapour of 12% ethanol. The data showed an analogous cline, the alcohol tolerance decreases the towards equator. The direction of the alcohol tolerance cline is the same as the Adh^F cline. But there is no clear correlation between the alcohol tolerance of natural populations and the frequency of Adh^F. Briscoe *et al.*, (1975), Hickey and Mclean (1980) found that the winery populations in Spain and Canada had a higher Adh^F frequency than surrounding populations not exposed to ethanol. However such a relationship was not found by other authors. McKenzie and Parsons (1974), McKenzie and McKechnie (1978) and Marks *et al.* (1980) measured Adh^F frequency and tolerance to ethanol in winery populations and compared it to nonwinery populations in Australia and California, but found no increase in Adh^F frequency. A similar conclusion was reached by some work on laboratory populations. Gibson *et al.* (1979) successfully selected for increased tolerance to ethanol in polymorphic laboratory populations, but found no consistent increase in Adh^F frequency nor in ADH activity.

Aquadro *et al.* (1986) and Scott *et al.* (1988) reported that molecular changes found in flies from natural population in an 11.8kb region of *Adh* gene affected *Adh* gene expression. Aquadro *et al.* (1986) found that a line with a complete 5.2kb *copia* insertion approximately 400bp 5′ to the distal promoter showed a developmental stage specific effect, with larvae having much less ADH activity than adults. Scott *et al.* (1988) proposed that a 4.5kb insertion approximately 3kb 5′ to the distal promoter may be responsible for the decrease of ADH molecules and lower ADH activity in adults in a regulatory mutation line.

All of these data suggest that insertions and deletions in the region of the *Adh* gene affect ADH activity in natural population of *Drosophila melanogaster*. I have studied ADH activity in second isochromosome strains extracted from the Australian and Chinese populations along the *Adh* cline spanning a large geographic range in both hemispheres. The aims are to see:

(1) whether there is variation in ADH activity between the populations,

(2) whether populations at similar latitudes have similar ADH activity level,

(3) whether the ADH activities of Adh^F and Adh^S alleles are similar in the Australian and Chinese populations,

(4) are there any nonrandom associations between restriction endonuclease variants and ADH activity.

To answer these questions I have investigated ADH activity in 104 lines from seven Australian and 88 lines from six Chinese populations. Also for this study I chose 52 strains with either high or low ADH activity from ADH-F and ADH-S lines and six strains of ADH-FChD from the Australian and Chinese populations. In these strains I investigated the levels of ADH protein to test the relationship between ADH protein amount and ADH activity. As restriction maps of these strains were available it was possible to study the relationship between restriction endonuclease variation in the *Adh* gene region and the expression of the *Adh* gene.

6.2 Materials and Methods

6.2.1 ADH activity assay (follows Gibson *et al.*, 1980)

The *Drosophila melanogaster* second isochromosome lines assayed for ADH activity were the same lines used for restriction mapping (see chapter 3) except two lines from the Guanghzhou population (G5 and G14) which had been lost. These lines are homozygous for wild-type second chromosomes, wild-type third chromosome and X chromosome from second-third chromosome translocation stock $T(2;3)ap^{Xa}$. The wild-type second and third chromosome vary between lines, the effects of modifier loci located on these two wild-type chromosomes can be detected in assays.

Two replicate cultures, each with twenty pairs of male and female flies from each line, were set up in 250ml plastic bottles on standard culture medium (see Chapter 2) at 25°C. From the progeny male flies were aged for 5-7 day and then etherized, weighed and homogenised in glass grinders on ice in cold 100mM sodium phosphate buffer (pH7.5) to a final concentration of 10mg/ml live weight. The homogenates were centrifuged at 10,000rpm (Sorvall SM-24 rotor) for 30 minutes. The supernatants were kept on ice until assayed. Two sets of flies were collected from each replicate culture. Flies from a line(C5) homozygous for Adh^F and a line(C8) homozygous for Adh^S were prepared in the same way and used as controls in the assays. These two lines were originally derived from the Cygnet, Tasmanian population and have previously been used as controls in a number of experiments (Freeth, 1988).

ADH activity was measured in a 1ml reaction mixture of 150mM isopropanol and 2 mM NAD in 100mM sodium phosphate buffer (pH7.5) with 10ul (for ADH-F and ADH-FChD lines) or 20ul (for ADH-S lines) of

extract. The reaction was monitored at 340nm to record NADH production for 2-3 minutes on a Gilford 250 spectrophotometer. Each assay was repeated twice. One unit of ADH activity was defined as an increase in absorbance at 340nm of 0.001/min at 25°C, ie 1.61×10^4 nmoles NADH produced/minute. Enzyme activity was expressed as units per milligram live weight and was termed activity units. The heat stability of the extracts was tested by keeping them in a 40°C water bath for 5 minutes before assaying for ADH activity. Ethanol was also used as a substrate in the same experiments so that the ratio of activity with 2-propanol to activity with ethanol could be calculated (Gibson *et al.*, 1980; Chambers *et al.*, 1984)

6.2.2 Radial immunodiffussion (follows Lewis and Gibson, 1978)

52 high or low ADH activity lines from the Australian and Chinese populations were chosen to test the relationship between ADH activity and the amount of ADH protein, using radial immunodiffusion (Lewis and Gibson, 1978). These lines were selected because they showed extremely high or low ADH activity in the populations in which they occurred. A 1.5% agarose gel was made in 100 mM sodium phosphate buffer (pH7.5) with 2.5% crude ADH antisera. The antisera made available to me was obtained after injecting rabbits with crude extracts of flies homozygous for Adh^F (Lewis and Gibson, 1978; Freeth *et al.*,1986). Samples were prepared in the same way as for the ADH activity assays (6.2.1). Ten µl of extract was added to each well (diameter=3mm) punched in the gel. Each sample was loaded into two wells. Immunodiffusion was carried out for 40 hours at 4°C and the ADH/anti-ADH immunoprecipitate was stained specifically with the ADH stain used after electrophoresis (see chapter 2). Dilution series (100, 50, 25, and 12.5) of C5 (ADH-F) and C8 (ADH-S) were run on each gel in the

first row. The result of this standard series was found to be comparable between gels with a least square regression line fitted to diameter versus log concentration plots. All sample protein concentrations (expressed as arbitrary protein units) were determined from these regression lines and are relative to the standard series for each gel (see Figure 6.3.3).

6.3 Results

The ADH activity of each single second chromosome line from the Australian and Chinese populations is shown in Figure 6.3.1 a ,b. The level of ADH activity in these lines is spread over a ten fold range. One line (Ch14) from the Coffs Harbour population was found to be a null activity allele, it is excluded from the statistical analyses. The overall averages of ADH-F and ADH-S activity in the Australian and Chinese populations are shown in Table 6.3.1.

The average ADH activity, overall, in the Chinese populations is significantly higher than in Australian populations (P less than 0.001). In both the Australian and the Chinese samples the ADH activity of the lines fall mainly into two groups: the Adh^F lines have on average more than twice the activity of the Adh^S lines, although there is some overlap between the two groups. The ranges of ADH activity of the Adh^F lines varies from 35.4 to 351.9 in the Australian populations and from 130.7 to 365.7 in the Chinese populations. The range of ADH activity of the Adh^S lines varies from 36.1 to 179.5 in the Australian populations and from 30.3 to 190 in the Chinese populations. The average levels of Adh^F and Adh^S activities are higher in China than in Australia, and the statistical analysis (t-test) indicates the differences are significant (P less than 0.01 for ADH-S, P less than 0.001 for ADH-F).

The ADH activities in six lines homozygous for Adh^{FChD} are very similar (129, 131.25, 132.9, 138.6, 145.3, and 189.5). The average ADH activity of Adh^{FChD} lines is 144.4±9.3, which is significantly different from the averages of Adh^{F} and Adh^{S} lines ($P < 0.01$).

The mean ADH activities of Adh^{F} and Adh^{S} lines in each population are shown in table 6.3.2. They vary between populations. In the Australian populations the means of ADH-F activity vary from 174.9 to 255.9, the means of ADH-S activity vary from 55.5 to 116.5; in the Chinese populations the means of ADH-F activity vary from 185.6 to 280.7, the means of ADH-S activity vary from 50.3 to 146.6. The mean activity of ADH-F in the Shanghai population (280.7) is significantly higher than in Chateau Yarrinya ($T_{17}=3.2$, $P < 0.01$), All Saints ($T_{14}=3.01$, $P < 0.01$), Jinan ($T_{18}=3.01$, $P < 0.01$) and Haikou ($T_{15}=3.48$, $P < 0.005$). The mean activity of ADH-S in Coffs Harbour (116.5) is significantly higher than that in Chateau Yarrinya ($T_{11}=3.4$, $P < 0.01$). The mean activity of ADH-S in Haikou (128.4), Guangzhou (146.4) and Fuzhou (135) is significantly higher than that in Shanghai ($T_{10}=5.58$, $p < 0.001$; $T_{8}=6.03$, $P < 0.001$; $T_{8}=3.3$, $P < 0.02$, respectively), Jinan ($T_{9}=8.74$, $P < 0.001$; $T_{7}=9.27$, $P < 0.001$; $T^{7}=5.09$, $P < 0.002$) and Dalian ($T_{7}=5.15$, $P < 0.001$; $T_{5}=5.48$, $P < 0.005$; $T_{5}=2.68$, $P < 0.05$) populations.

Overall the variation in the activities of the Adh^{F} lines is not related to latitude, but that of Adh^{S} lines does show some geographic differentiation, particularly in the Chinese populations. The correlation of mean ADH-S activity in the Chinese populations on latitude is significant (correlation coefficient in linear regression of ADH activity on latitude is -.89, P less than 0.01). The mean activity of Adh^{S} lines decreases with distance from the equator. For the Australian populations the correlation coefficient of ADH-S

activity on latitude is -.39, and it is not statistically significant. If the data from the island of Tasmania are excluded, because Adh^S frequencies in Tasmanian populations are unexpectedly high compared to elsewhere at the same latitudes (see chapter 5), then the correlation coefficient is high and has the same sign (-.62) as that of Chinese populations, although it is still not statistically significant (see table 6.3.3). ADH protein was measured by radial immunodiffusion in 52 high or low ADH-F and ADH-S lines from each population plus ADH-FCHD lines and one null activity line. In total there were 27 ADH-F lines, 25 ADH-S lines, six ADH-FChD lines. Figure 6.3.2 shows the ring diameters of each measured line which were transformed into arbitrary units of ADH protein using the least-squares regression line derived from a series of standard dilutions (figure 6.3.3).

The ring diameters vary between strains. The plots of ADH activity against ADH protein (Figure 6.3.4.a,b.c,d) show significant linear regression relationships between ADH activity and the amount of ADH protein both for Adh^F and Adh^S lines. The correlation coefficients are similar, 0.73 (P less than 0.01) for Adh^F lines, and 0.69 (P less than 0.01) for Adh^S lines. Overall strains, including Adh^F, Adh^S and Adh^{FChD}, the relationship is also significant (0.69, $P < 0.01$, see table 6.3.4). For the null allele (Ch14) strain there is no stained ring, comfirming that no ADH protein is produced by this strain. The relationships between ADH activity and ADH protein are shown in figure 6.3.4 a,b,c,d; the least-squares linear regression lines are also drawn. Regression equations and correlation coefficients are given in table 6.3.4.

The data from the single radial immunodiffussion experiments show that on average the Adh^F strains possess at equilibrium a significantly (P less than 0.01) higher number of molecules of ADH

protein than *Adh*^{S} strains (74.5±3.9 vs 56.2±2.7) (Figure 6.3.4.c). This difference partly accounts for the difference in their ADH activities. The mean ADH activity of *Adh*^{F} lines in this radial immunodiffussion experiment is 209.9±16.4, twice as high as that of *Adh*^{S} lines (90.2±8.7).

The ADH activity and the amount of ADH protein in the *Adh*^{FChD} lines are similar to each other. The ADH activities of these thermostable strains had a mean of 144±9.3, which is significantly different from that of *Adh*^{F} lines (P less than 0.05) and *Adh*^{S} lines (P less than 0.01); although one strain had an exceptional higher level of activity (189.5). The mean levels of ADH protein of these thermostable lines is 64±1.6, which is intermediate, but not significantly different from that of *Adh*^{F} or *Adh*^{S} lines. (Figure 6.3.4.c and table 6.3.4)

To test whether these Chinese thermostability *Adh* variants encode an enzyme with similar properties to ADH-FChD (which has similar biochemical properties to the enzyme encoded by two other thermostability *Adh* variants, ADH-Fr and ADH-71K, Gibson *et al.*, 1980), single second chromosome strains of heat resistant and heat sensitive alleles from three Chinese populations were compared.

The activity ratio (seconary/primary alcohol activity ratio, which differs between *Adh*^{F} and *Adh*^{S} lines) and heat stabilities of the ADH enzymes encoded by the three Chinese thermostability alleles were very similar to those previously shown to be characteristic of ADH-FChD but different to the control ADH-F (Gibson *et al.*, 1980). (Table 6.3.5). The extracts of thermostable lines show 80% to 100% of the ADH activity remained after the extracts were kept in a 40^{o}C water bath for 5 minutes, however only 50%-60% of ADH activity remained in the heat-sensitive lines.

The results of ADH activity assays on extracts of these homozygous strains (table 6.3.4), using ethanol and 2-propanol as substrates showed that the ratios of activity with 2-propanol to activity with ethanol were all close to 4.5 in the four Chinese ADH thermostable lines. This ratio is similar to the control ADH-FChD (4.2) but different from that found for all heat-sensitive Adh^F alleles, in which the ratios are around 7.0. An exceptional heat sensitive strain from the Jinan population differed in activity ratio (5.2). This variant merits further investigation.

The ADH activity data for the second isochromosome lines extracted from the Australian and Chinese populations were analysed in relation to the restriction endonuclease variation found in the *Adh* region in the same lines. Thus it is possible to test whether any of the restriction map variation affects ADH activity. The relationship between ADH activity and restriction map variation was investigated by comparing ~~of~~ ADH activity between lines with and without different restriction endonuclease variants, and by testing for associations between the lines showing exceptional ADH activity and the restriction endonuclease variants.

For the first comparison variants with frequencies less than 10% or greater than 90% were excluded from the analyses for statistical reasons. The results are given in table 6.3.5.a, b. Five restriction site variants, *Bam*HI(-7.2), *Hin*dIII(-3.7), *Hin*dIII(-3.0), *Pst*I(1.3) and *Eco*RI(9.0), are associated with significant changes in ADH activity. In Australia, the ADH activity of Adh^F lines with variants *BamHI(-7.2), and HindIII(-3.7)* decreased, and ADH activity of Adh^F lines with *EcoRI(9.0)* increased, ADH activity of Adh^S lines with *Pst*I(1.3) decreased. In China, the ADH activity of Adh^S lines without

*Bam*HI(-7.2) and with *Hin*dIII(-3.0) increased. But none of the variants show consistent effects on ADH activity on both continents.

For the insertions/deletions the analyses showed only one deletion (0.2) was associated with an increase of ADH activity in the Adh^S line in China. But this variant did not show a consistent effect on ADH activity in Adh^S lines in both countries.

The two lines with the highest and lowest ADH-F and ADH-S activity from each population, and the restriction endonuclease variants found in these lines are listed in table 6.3.7.a,b. There is no consistent relationship between deletion (0.2), insertion (0.4) and ADH activity. There are a number of points to be noted: (1) only two out of eleven high ADH-S activity lines carry *BamHI(-7.2)* in Chinese populations, although the frequency of this variant in the Chinese Adh^S alleles is 0.4. Five out of 14 low ADH-F lines carry *BamHI(-7.2)* in the Australian populations and for one of them (line C.Y.13) the ADH-F activity dropped to 35.4. No insertions or deletions were found in this line. The *BamHI(-7.2)* is usually associated with the Adh^S allele, and recombination seems to affect ADH activity. (2) The majority of the insertions and deletions had slight effects on ADH activity; such as I(0.4). I(0.7) and D(0.2). However, some large insertions in the *Adh* gene region greatly decrease ADH activity, such as lines A113, and H4. The 5.0kb insertion in intron 1 of A113 (ADH-F) is the likely cause of the dramatic decrease (to 43) in activity in this line. ADH activity of line H4 (ADH-F), which has 1.5kb insertion about 2kb from the 5′ end of the coding region, dropped to 132.9, the lowest level of ADH-F activity, and a similar level to ADH-S activity in this population (the mean ADH-F activity is 185.6 and the mean ADH-S activity is 128.4 in the Haikou population). (3) Line T40 has a

duplication (see figure 3.3.2) and this line showed the highest ADH-F activity of lines in the Tamar population.

6.4 Discussion

Assays of ADH activity in the Australian and Chinese populations show that ADH activity is distributed continuously, although, as previously reported (Gibson and Miklovich, 1971; Birley and Barner, 1973, 1975; Kamping and Van Delden, 1978), there is a strong association between the fast allele and high activity, and the slow allele and low activity. In the present data, ADH activity in natural populations was spread over a tenfold range, which is similar to the data of Anderson and Gibson (1985), but higher than that from previous reports by Aquadro *et al.*,(1986) and Birley and Marson(1981) (fivefold). Laurie-Ahlberg *et al.* (1980) reported that the ratio of highest to lowest line mean ADH activity of male flies in an American population exceeded 13. However the mean ADH-F activity is twice as high as the mean ADH-S activity in Australian and Chinese populations, which is consistent with previous reports.

The level of ADH activity varies between different geographic regions. The mean ADH activity of Adh^F and Adh^S lines in China are significantly higher than in Australia, and also the level of ADH activity varies between different Chinese or Australian populations (table 6.3.1 and table 6.3.2). The distribution of ADH-F activity between populations seems quite random, but the distribution of ADH-S activity in China shows a latitudinal cline, with the mean ADH-S activity decreasing with increasing latitude. This cline is parallel to the cline in the frequency of the restriction endonuclease variant, deletion (0.2) (see chapter 3). The study of the relationship between ADH activity and restriction endonuclease variants (table 6.3.6.b)

shows that the mean ADH activity of *Adh*^{S} lines with deletion (0.2) is significantly higher than that of *Adh*^{S} lines without deletion (0.2) (P less than 0.001) in Chinese population. It is possible that the deletion (0.2) is responsible for the cline of ADH-S activity in China. In Australian populations there are not enough *Adh*^{S} lines with deletion (0.2) (only two) to make a comparable analysis. It will be interesting to investigate whether the effect exists in other geographic regions.

A null activity allele (Ch14) was found in the Coffs Harbour population. The haplotype of this null allele is different from that of the Tasmania null alleles (See Chapter 5). This null allele carries an exceptional restriction site *Eco*RI(1.4). This site is probably not responsible for the loss of ADH activity, since Gibson *et al.*, (unpublished data) found that there is a 440bp deletion in exon 2, which is more likely to be the cause of the null activity (see chapter 4).

The source of ADH activity variation, according to published work, may be due to the differences in the amount and catalytic efficiency of the ADH protein produced by the allele. Gibson (1972) investigated the amount of ADH protein in *Adh*^{F} and *Adh*^{S} lines on a common genetic background and found that *Adh*^{F} lines contained roughly twice as many ADH molecules as *Adh*^{S} lines. Later Lewis and Gibson (1978) investigated eight *Adh*^{F} lines, seven *Adh*^{S} lines and one *Adh*^{FChD} line extracted from Australian winery populations (Mudgee and Scone). They found that *Adh*^{F} lines on the whole have higher ADH activity and number of ADH molecules, and enzyme extracted from *Adh*^{F} lines hase higher mean catalytic efficiency per enzyme molecule than enzyme extracted from *Adh*^{S} lines (1:0.82). The *Adh*^{FChD} lines show an intermediate level of ADH activity and ADH protein. These results have been confirmed by

McDonald *et al.*, (1980), Anderson and McDonald, (1983) and Clarke and Whitehead, (1984). My data (table 6.3.4) is consistent with these data. In total 27 Adh^F lines from Australian and Chinese populations possess higher mean ADH activity (twice) and number of ADH molecules (1:0.75) than 25 Adh^S lines. The ratio of mean catalytic efficiency per enzyme molecule extracted from Adh^F and Adh^S lines is 1:0.58. This result suggests that the difference in catalytic efficiency of the ADH enzyme may account for about 2/3 of the difference in ADH activity between Adh^F and Adh^S lines. Winberg *et al.* (1985) measuring active ADH found that the Adh^F allele enzyme has a catalytic-center activity for secondary alcohols that is four times larger than that of Adh^S, and this is consistent with a stronger binding of the reduced coenzyme to Adh^S, and hence a slower dissociation of the coenzyme from the binary enzyme-NADH complex with Adh^S, causing slower hydride transfer. With ethanol the catalytic-center activity of Adh^F is twice that of Adh^S. The single amino acid substitution at position 192 distinguishing the two allelozymes is believed to be mainly responsible for the difference of catalytic-center activity between the two allozymes (Winberg *et al.*,1982, Winberg *et al.*,1985 and Laurie-Ahlberg and Stam, 1987).

The statistical analyses in this study (table 6.3.6.a,b. and table 6.3.7.a,b,c,d) suggest that there is no consistent relationship between ADH activity and restriction endonuclease variation, although there are some exceptions. This result is similar to that of other authors. Aquadro *et al.* (1986) found that most of the restriction endonuclease site variants showed little effect on ADH activity, and although eighty percent of the lines studied were shown to have at least one insertion or deletion, there was little evidence of any effect on adult ADH activity level. One exception was a complete copia

insert approximately 400bp 5′ to the distal promoter which caused a large loss of ADH activity in the larval stage. Insertions/deletions are usually mildly deleterious, but when large insertions/deletions occur in functionally critical regions ADH activity is greatly changed. In this study an Adh^F line A113 has a 5.0kb insertion in intron 1, and ADH activity dropped to 43 and the amount of ADH protein decreased to 20.0. Intron 1 is 654 base pair long and is between the adult leader sequence and larval leader sequence. It is removed by splicing to give the mature adult mRNA (Benyajati *et al.*, 1983). Further study (Gibson and Cao unpublished data) indicates the ADH activity of this line is normal in the larval stage. It is likely that the 5.0kb insertion in intron 1 affects the splicing of adult mRNA, but has no effect on larval mRNA. Another line, T40, which has a duplication in the region of *Adh* gene, possesses the highest ADH activity in the Tamar population, the duplication includes the 5′ part of the coding region where the promoters are located. Double promoters may enhance ADH activity.

The statistical analyses show that the *Bam*HI(-7.2) site variant, which is usually associated with the Adh^S genotype, may affect ADH activity: in Australia the mean ADH activity of Adh^F lines with this variant is significantly lower than average; in China the mean ADH activity of Adh^S lines without this variant is significantly higher than average (see table 6.3.6.a). Five low ADH-F activity lines in Australian populations were found to be associated with the restriction site variant BamHI(-7.2) (see table 6.3.7.b). For example the ADH activity of C.Y13 (Adh^F line) carrying the *Bam*HI(-7.2) site was 35.4, and the amount of ADH protein decreased to 53.5 units. This form of recombination affecting ADH activity was also reported by Aquadro *et al.*,(1986). They found two lines with the "wrong" ADH

activity (NC16, Slow, but high activity without the *BamHI(-7.2)* site; KA12, Fast, but low activity with the *BamHI(-7.2)* site), and both lines appear to be recombinants. Nonrandom association between the *BamHI(-7.2)* site and Adh^S was found in most of the Australian and Chinese populations and has been reported in American populations (Langley *et al.*, 1982; Aguadro *et al.*, 1986), and a European population-Groningen (Cross and Birley, 1986). Amazingly the association between *Bam*HI(-7.2) and Adh^S was seldom found in the southern Chinese populations (none in the Haikou population, three out of six in the Guangzhou, and one out of five in Fuzhou (see table 3.3.1). Since the southern Chinese populations show quite high ADH-S activity, recombination in the region between the allozyme and *Bam*HI(-7.2) site may include modifiers which enhance or reduce the ADH activity. The latitudinal cline of ADH-S activity in Chinese populations may also be related to this recombination event. This suggests that these modifiers may be tightly linked to the threonine-lysine replacement which distinguishes Fast and Slow.

According to the analyses in this chapter and previous work ADH activity in natural populations depends mainly on the Adh^F frequency in the population, since on average ADH activity produced by the Adh^F allele is twice as high as that produced by the Adh^S allele. A cline in ADH activity in natural populations of *Drosophila melanogaster* parallel to the cline of Adh^F frequency is therefore expected and observed. Also none of the restriction endonuclease variants found consistently effect ADH activity or show consistent latitudinal clines in Australian and Chinese populations.

The study of Chinese Adh^F heat resistant alleles, which occur in southern China at high frequency, support the argument that the heat resistant alleles found in southern Chinese populations are similar to

Adh^{FChD} and may have originated in southern China. The lines have similar ratios of activity with 2-propanol to activity with ethanol as substrate, but different ratios from that of *Adh*^{F} (table 6.3.5). The variation in ADH activity and ADH protein levels of the thermostable alleles are smaller than those of *Adh*^{F} and *Adh*^{S}, (Figure 6.3.4c). This suggests a relatively recent occurrence of *Adh*^{FChD}.

Gibson *et al.*,(in press) used two allele specific oligonucleotide probes, 5'CCC ACC CAG CCC TCG TTG G 3', containing the triplet CCC which corresponds to proline at residue 214 in *Adh*^{F} (probe AF), and 5' CCC ACC CAG TCC TCG TTG G 3' containing triplet TCC which corresponds to serine at the same residue in *Adh*^{FChD} (probe AFD), to hybridize to the 368 bp region of *Adh* gene which includes the nucleotides specific for the proline or serine residue. DNA, amplified by the polymerase chain reaction, from eleven thermostable *Adh* alleles extracted from populations in north America, Europe, Australia and China all hybridized to probe AFD, but not to probe AF. The probe AF only hybridized to DNA extracted from control *Adh*^{F} alleles. Both probes did not hybridize to the DNA extracted from control *Adh*^{S} alleles.

The above is strong evidence that the thermostable mutations are molecularly the same, and that they had a common origin, probably originating in southern China where high frequencies of *Adh*^{FChD} were found.

Table 6.3.1 Mean ADH activity in Australian and Chinese populations.

	Australian	Chinese	t-test (degree of freedom) of difference
overall	151.0 (n=103) (7.3)	197.2 (n=82) (9.3)	3.98*** (184)
Adh^F	200.1 (n=60) (7.4)	243.4 (n=54) (7.8)	4.02*** (110)
Adh^S	87.4 (n=43) (5.1)	111.9 (n=28) (8.6)	2.79** (72)

** $p < 0.01$, *** $p < 0.001$. n is the number of lines measured for ADH activity. Standard errors are given in parentheses below the means.

Table 6.3.2 Mean ADH-F and ADH-S activities in Australian and Chinese populations. Standard errors are given in parentheses. n is the number of samples.

Australia						China					
population	latitude	ADH-F	n	ADH-S	n	population	latitude	ADH-F	n	ADH-S	n
Cygnet	43°09′S	200(12.7)	8	91.5(9.5)	7						
Tamar	41°02′S	194.7(11.1)	10	78.4(7.9)	5						
						Dalian	38°54′N	255.9(13.1)	13	69(7.0)	2
Chateau Yarrinya	37°36′S	174.9(26.8)	9	55.6(9.5)	6						
						Jinan	37°N	190.5(14.8)	10	50.3(7.0)	4
All Saints	36°03′S	204.3(18.9)	11	63.5(12.6)	4						
Araluen	35°39′S	225.6(20.4)	10	71.2(18.9)	5						
						Shanghai	31°10′N	280.8(19.9)	10	74(9.6)	5
Coffs Harbpur	30°18′S	180(10.6)	6	116.5(15.8)	7						
						Fuzhou	26°05′N	246(7.4)	9	135(15.8)	5
						Guangzhou	23°08′N	220.9(48.8)	6	146.6(10.5)	5
						Haikou	20°20′N	185.6(15.2)	6	128.4(6.1)	7
Cardwell	18°16′S	225.9(18.8)	6	94.5(5.2)	9						

Table 6.3.3 Correlation coefficents of ADH-F and ADH-S activity on latitude.

	ADH-F activity	ADH-S activity
Australian populations	r=-0.30(ns) (n=7)	r=-0.39(ns) (n=7)
Australian populations without the data from the Tasmanian populations	r=-0.45(ns) (n=5)	r=-0.62(ns) (n=5)
Chinese population	r=0.14(ns) (n=6)	r=-0.89** (n=6)

** $p<0.01$, ns-not significant.

Table 6.3.4 Mean ADH activity, mean ADH protein amount and the regression equations describing the relationships between protein and activity in the strains measured. Standard errors are given in parethesis.

Genotype class	No. of strains	mean ADH activity	mean ADH protein amount	least-squares regeression line	Correlation coefficient
Adh^F	27	206(16.4)	74.5(3.9)	Y = -22.7+3.07X	.73**
Adh^S	25	90.2(8.7)	56.1(5.1)	Y = 7.24+1.37X	.69**
Adh^{FChD}	6	144.4(9.3)	64(1.6)	Y = 348.2-3.18X	-0.53(ns)
overall	58	149.7(11.2)	67.4(2.7)	Y = -38.6=2.8X	.69**

** $p < 0.01$

Table 6.3.5 Comparison of ADH activities (standard errors in parenthesis) and thermostabilities in extracts derived trom heat sensitive and heat resistant *Adh*^{F} alleles extracted from the P. R. C populations.

origin of allele	ADH activity with propanol	Activity ratio*	ADH activity remaining in 40^{o}C heat-treated extracts (%)
Heat-resistant *Adh*^{F} alleles			
Control *Adh*^{FChD}	131.3(1.8)	4.2	94.6
Guangzhou	130.0(2.0)	4.2	95.8
Guangzhou	103.5(1.2)	4.0	81.2
Fuzhou	190.5(1.5)	4.9	80.7
Jinan	107.5(5.5)	4.5	100
Heat-sensitive *Adh*^{F} alleles			
Control *Adh*^{F}	195.1(6.0)	6.8	59.7
Guangzhou	197.0(small)	7.3	55.1
Fuzhou	210.1(small)	6.7	51.8
Jinan	209.5(0.5)	5.2	53.6

* Ratio of activity with 2-propanol to activity with ethonal as substrate. Control *Adh*^{FChD} and *Adh*^{F} alleles are described in Gibson *et al.*, 1980.

table 6.3.6.a Comparisons of ADH activity between the lines with or without restriction endonuclease site variants. n is the number of the lines measured, k is the number of the lines carrying the restriction endonuclease variant. Standard errors are given in parantheses. + and - denote the presence or absence of the variant respectively.

	Australia						China					
		Adh^F (n=58)			Adh^S (n=46)			Adh^F (n=54)			Adh^S (n=28)	
	k	+	-	k	+	-	k	+	-	k	+	-
B(-7.2)	15	175.5*	210	35	87.9	85.8				11	87.5**	127.5
		(13.0)	(8.5)		(5.2)	(15.2)					(14.7)	(8.6)
h(-6.9)				5	104.9	85.3						
					(17.2)	(5.3)						
H(-3.7)	6	155.5**	206.1									
		(29.5)	(7.1)									
H(-3.0)	12	191.1	203.4	12	88.5	87.0				5	140*	105
		(10.5)	(8.9)		(11.7)	(5.6)					(10.1)	(9.9)
P(1.3)				9	60.4**	93.9				7	121.6	108.6
					(6.7)	(5.7)					(17.2)	(10.1)
E(9.0)	9	240.9*	192	10	84.2	88.4	7	239.9	244.6	5	136.3	106.4
		(16.0)	(7.8)		(6.8)	(6.3)		(21.7)	(8.5)		(5.7)	(10.2)

* $p < 0.05$, ** $p < 0.01$. The abbreviations are: B - BamHI, h - HpaI, H - HindIII, P - PstI, E - EcoRI.

Table 6.3.6.b Comparison of ADH activity between lines with and without insertion/deletion. Abbreviations are as the same as in Table 6.3.6.a, I = insertion, D = deletion.

	Australia						China					
	Adh^F (n=58)			Adh^S (n=46)			Adh^F (n=54)			Adh^S S (n=28)		
	k	+	−	k	+	−	k	+	−	k	+	−
I(0.4)	6	179.3	202.6	9	73.5	91.0				7	121.6	108.4
		(8.6)	(8.1)		(9.6)	(5.8)					(7.2)	(10.1)
D(0.2)	13	187.8	204.6				29	249.7	238.8	13	147.7***	80.6
		(11.9)	(8.8)					(9.1)	(13.0)		(7.1)	(8.8)

Table 6.3.7.a The two lines with highest ADH activity amongst the Adh^F and Adh^S lines in each Australian population and the restriction endonuclease variants present. Activities of each line refer to figure 6.3.1.a.

ADH-F			ADH-S		
line	activity	variant	line	activity	variant
C11	high	B(-7.2)	C19	high	B(-7.2)
C51	high	E(9.0)	C28	high	h(-6.9)
T40	high	duplication	T50	high	B(-7.2)
T54	high	-	T33	high	B(-7.2), E(9.0)
C.Y.8	high	E(9.0)	C.Y.1	high	X9-7.4), B(-7.2), H(-3.0), P(1.3)
C.Y.9	high	B(-7.2), H(-3.7) H(-3.0)	C.Y.3	high	B(-7.2)
Al9	high	E(9.0)	Al1	high	X(-7.4), B(-7.2), P(1.3), I(0.28), I(0.4)
Al14	high	E(9.0)	Al7	high	B(-7.2)
Ar10	high	H(-3.0), D(0.2)	Ar1	high	B(-7.2)
Ar15	high	-	Ar3	high	B(-7.2), E(-9.0)
Ch5	high	H(-3.0)	Ch10	high	B(-7.2), H(-3.0), P(1.1)
Ch13	high	B(-7.2), P(1.3)	Ch12	high	B(-7.2)
Cd10	high	-	Cd6	high	B(-7.2), h(-6.9), H(-3.7), D(0.2)
Cd13	high	-	Cd8	high	B(-7.2), H(-3.0) E(9.0)

Table 6.3.7.b The two lines with the lowest ADH activity amongst the *Adh*^{F} and *Adh*^{S} lines in each Australian population and the restriction endonuclease variants present. Activities of each line refer to figure 6.3.1.a.

ADH-F			ADH-S		
line	activity	variant	line	activity	variant
C29	low	H(-3.0), D(0.2)	C45	low	-
C33	low	D(0.2), I(0.4)	C72	low	-
T29	low	h(-6.9), I(0.28)	T1	low	h(-6.9), P(1.3), B(3.9), I(0.4)
T56	low	B(-7.2)	T17	low	B(-7.2), B(3.9)
C.Y.10	low	H(-3.7), D(0.2)	C.Y.2	low	X(-8.0), B(-7.2), H(-3.0), P(1.3), I(1.0)
C.Y.13	low	B(-7.2), H(-3.7)	C.Y.5	low	B(-7.2), H(-3.0)
Al12	low	B(-7.2),	Al2	low	B(-7.2), H(-3.7)
Al13	low	I(5.0)	Al3	low	X(-7.4), B(-7.2), X(-5.3), P(1.3), I(0.4)
Ar6	low	-	Ar2	low	B(-7.2), E(9.0)
Ar12	low	-	Ar4	low	P(1.1), I(0.4)
Ch7	low	B(-7.2), H(-3.0). D(0.2)	Ch1	low	E(9.0)
Ch9	low	B(-7.2), H(-3.0)	Ch2	low	B(-7.2), H(-6.9), H(-3.0), P(1.1), B(3.9), I(0.4)
Cd11	low	H(-3.7), H(-3.0)	Cd1	low	H(-3.7), D(0.2)
Cd15	low	h(-6.9), P(1.3), B(3.9), E(9.0) I(4.5)	Cd13	low	B(-7.2), H(-3.0) E(9.0)

Table 6.3.7.c The two lines with the highest ADH activity amongst the *Adh*^{F} and *Adh*^{S} lines in each Chinese population and the restriction endonuclease variants present. Activities refer to figure 6.3.1.b.

ADH-F			ADH-S		
line	activity	variant	line	activity	variant
D6	high	H(-3.0)	-		
D17	high	-	-		
J1	high	H(-3.7)	J21	high	-
J11	high	-	J36	high	-
S7	high	X(-7.4), P(1.1) I(0.48)	S2	high	B(-7.2)
S39	high	H(-3.7), P(1.1), I(0.48)	S27	high	X(-7.3), P(1.3) I(0.4)
F2	high	D(0.2)	F28	high	D(0.2)
F10	high	-	F39	high	D(0.2)
G3	high	H(-3.0), E(9.0), D(0.2)	G10	high	B(-7.2), D(0.2)
G8	high	E(9.0)	G11	high	H(-3.0), P(1.3) I(0.4), D(0.2)
H1	high	E(9.0)	H12	high	H(-3.0), P(1.3) I(0.4), D(0.2)
H8	high	E(9.0), D(0.2)	H14	high	E(9.0

Table 6.3.7.d The two lines with lowest ADH activity amongst *Adh*^{F} and *Adh*^{S} lines in each Chinese population and the restriction endonuclease variants present. Activities of each lines refer to Figure 6.3.1.b.

ADH-F			ADH-S		
line	activity	variants	line	activity	variants
D20	low	X(-7.4), E(9.0) I(0.4)	-		
D30	low	E(9.0)	-		
J4	low	-	J14	low	B(-7.2)
J26	low	B(-7.2)	J16	low	B(-7.2), P(1.3), I(0.4)
S4	low	X(-7.4), I(0.4)	S28	low	-
S26	low	E(-8.6), D(0.2)	S24	low	B(-7.2)
F21	low	D(0.2)	F1	low	B(-7.2)
F23	low	H(-3.7), H(-3.0)	F33	low	H(-3.7)
G4	low	H(-3.0), D(0.2)	G9	low	B(-7.2), D(0.2)
G8	low	H(-3.0), E(9.0)	G15	low	B(-7.2), D(0.2)
H1	low	E(9.0)	H9	low	D(0.2)
H4	low	P(1.3),D(0.2) I(1.5)	H15	low	E(9.0)

The abbreviation for restriction endonuclease variants are same as Table 6.3.3 , for population are same as table 3.3.1. There are not enough *Adh*^{S} lines in Dalian population for analyses.

Figure 6.1 Diagrammatic representation of the *Adh* gene and the alternative transcription and splicing patterns for the predominant second instar larval and adult transcripts. Exons (ex) are shown as boxes and introns (in) as lines; the untranslated 5′ regions of exons are shown hatched while the 36-base region common to both transcripts is crosshatched. the sizes of the coding and noncoding regions are shown as base-pairs. (taken from Chia *et al.*, 1985 and modified by Freeth, 1986).

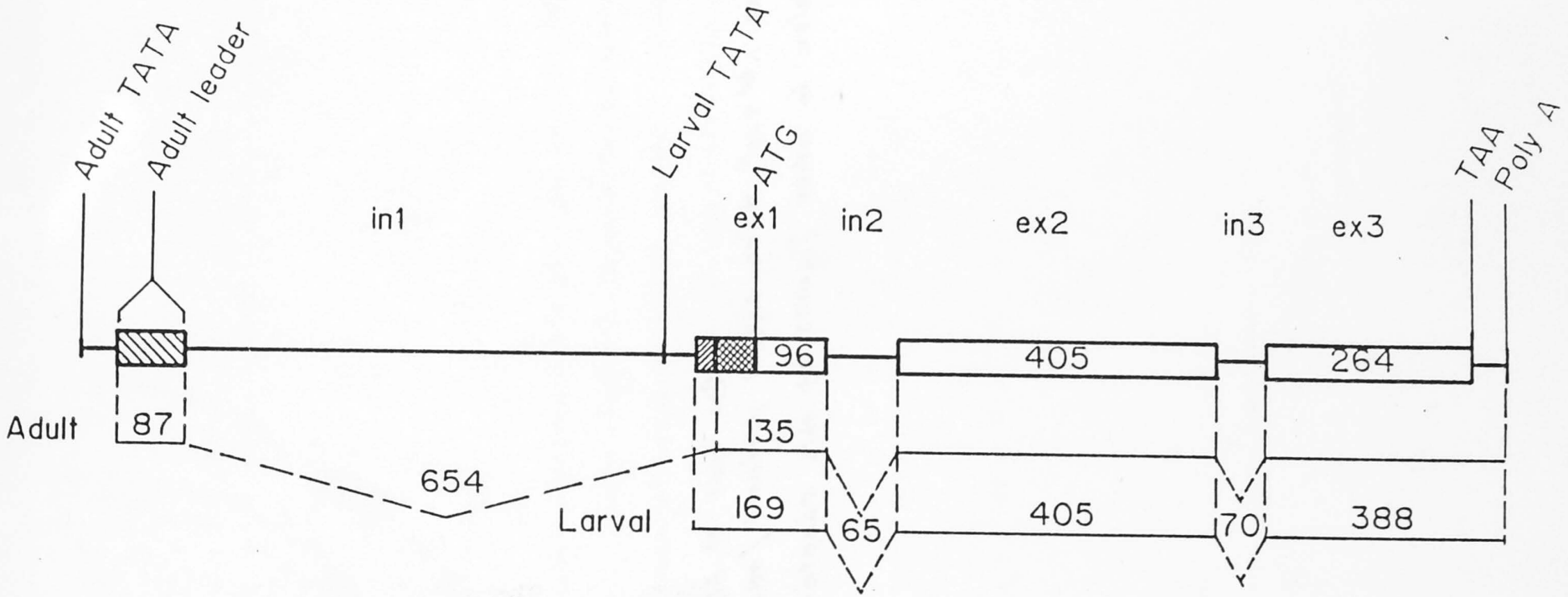

Adult TATA
Adult leader
Larval TATA
ATG
TAA
Poly A
in1
ex1
in2
ex2
in3
ex3
96
405
264
Adult
87
135
654
Larval
169
65
405
70
388

Figure 6.3.1.a The distribution of ADH activity in Australian populations of *Drosophila melanogaster*. A-Cygnet, B-Tamar, C-Chateau Yarrinya, D-All Saints, E-Araluen, F-Coffs Harbour, G-Cardwell. Open circle-Adh^F lines; filled circle-Adh^S lines. One unit of ADH activity was defined as 1.61×10^{-4} moles NADH produced / minute in this Figure and also in other Figures of this chapter.

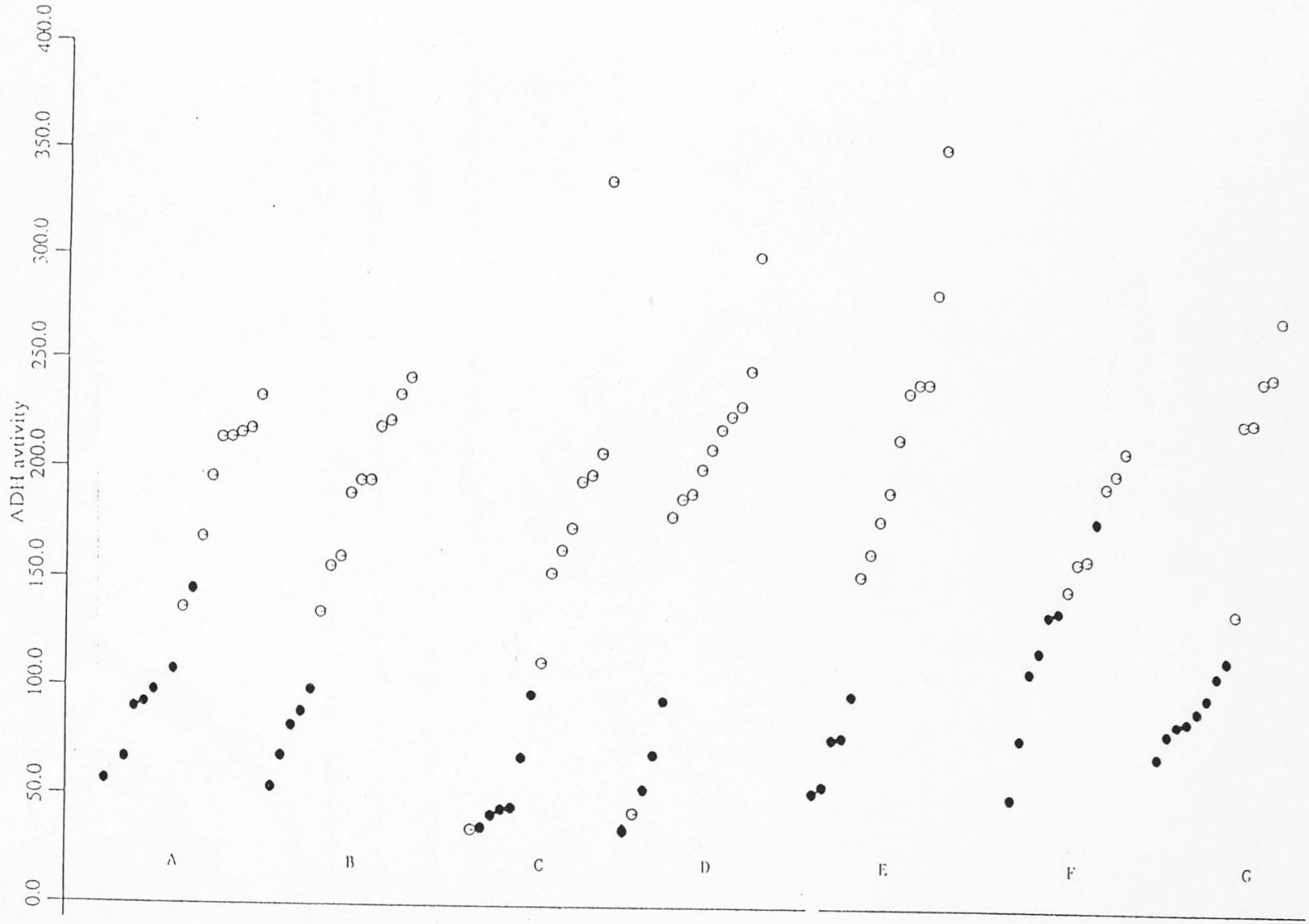

ADH avtivity
0.0
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0
A
B
C
D
E
F
G
populations

Figure 6.3.1.a

Figure 6.3.1.b The distribution of ADH activity in Chinese populations of *Drosophila melanogaster*. H-Haikou, I-Guangzhou, J-Fuzhou, K-Shanghai, L-Jinan, M-Dalian. Open circle-Adh^{F} lines; Filled circle-Adh^{S} lines; half filled circle-Adh^{FChD} lines.

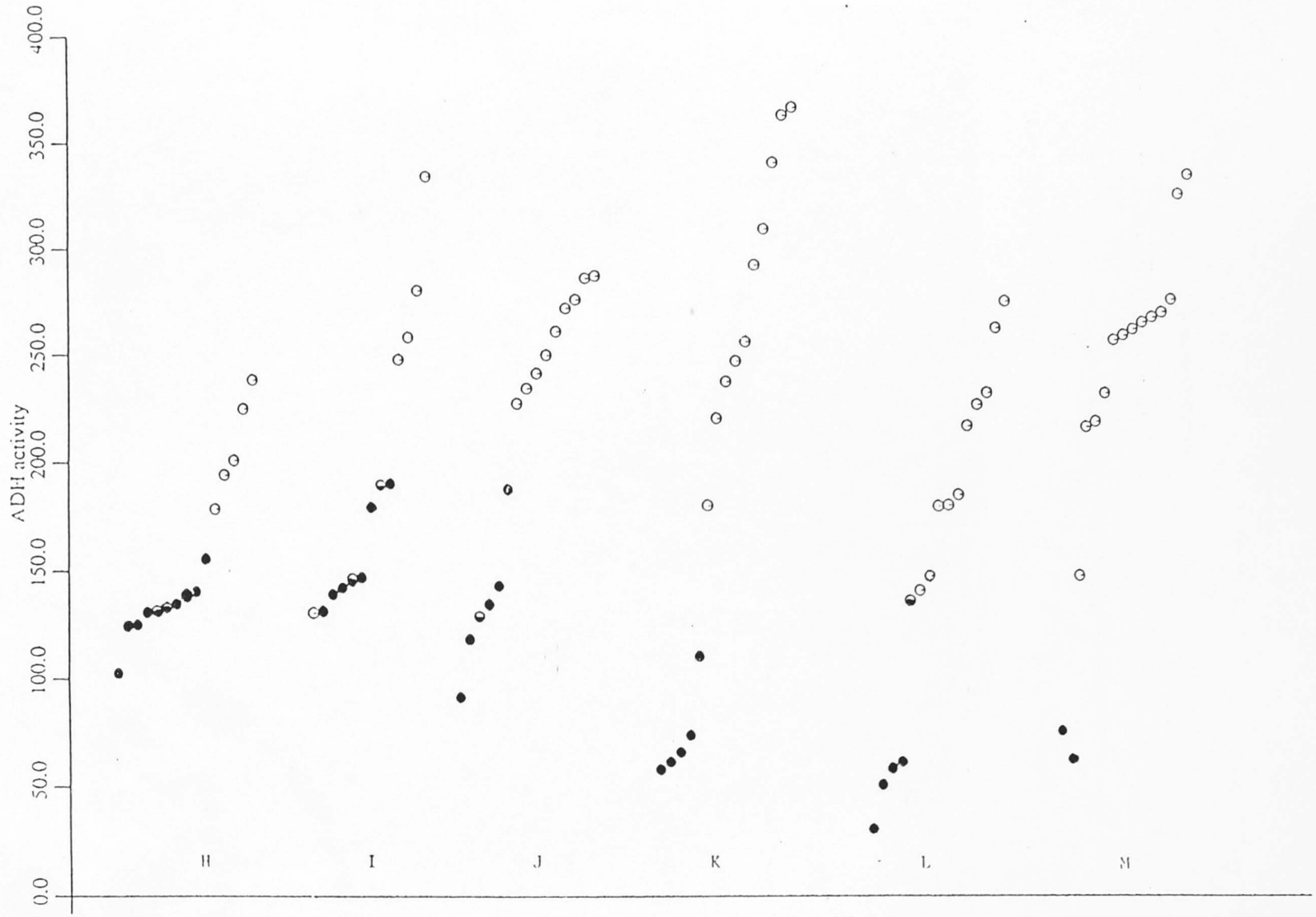

ADH activity
0.0
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0
H
I
J
K
L
M
population

Figure 6.3.1.b

Figure 6.3.2.a Single radial immunodiffusion plate after staining for ADH activity of high and low activity Adh^F lines from Australian and Chinese populations. The top row (left to right) is a dilution series of a crude extract from Adh^F control line C5, The largest circle, a 1:1 dilution of the crude extract, is called concentration 100; the next largest , a 1:3 dilution of crude extract, is called concentration 50; the next largest, a 1:7 dilution, is called concentration 25; the smallest, a 1:15 dilution, is called concentration 12.5.

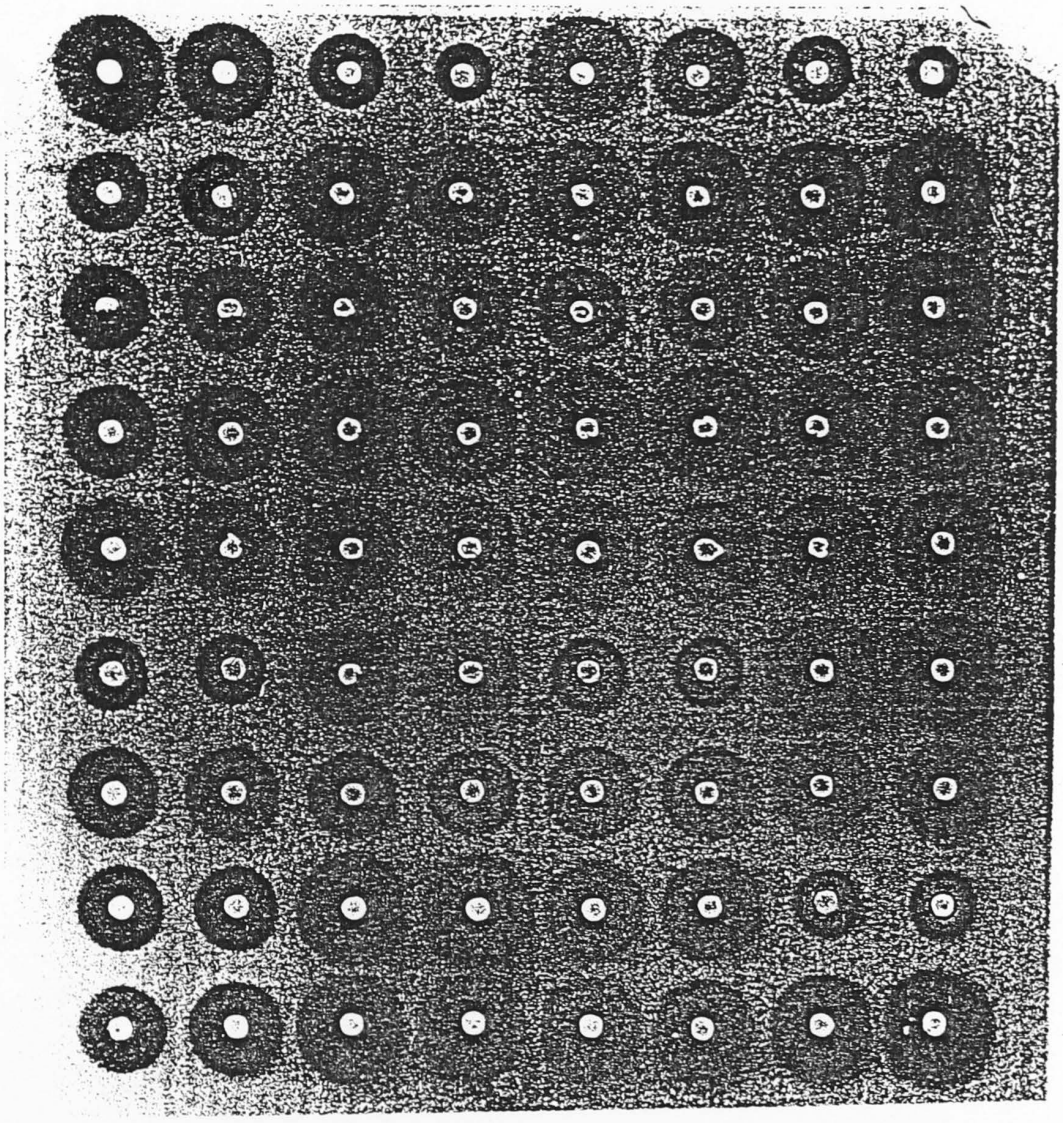


Figure 6.3.2.a

Figure 6.3.2.b Single radial immunodiffusion plate after staining for ADH activity of high and low activity *Adh*^{S} lines from Australian and Chinese populations. The top row is a dilution series of crude extract from *Adh*^{S} control line C8, which is diluted in the same way as C5. In the third and fourth column of the bottom row the extract of null activity line Ch14 was added, but no stained circles appear.

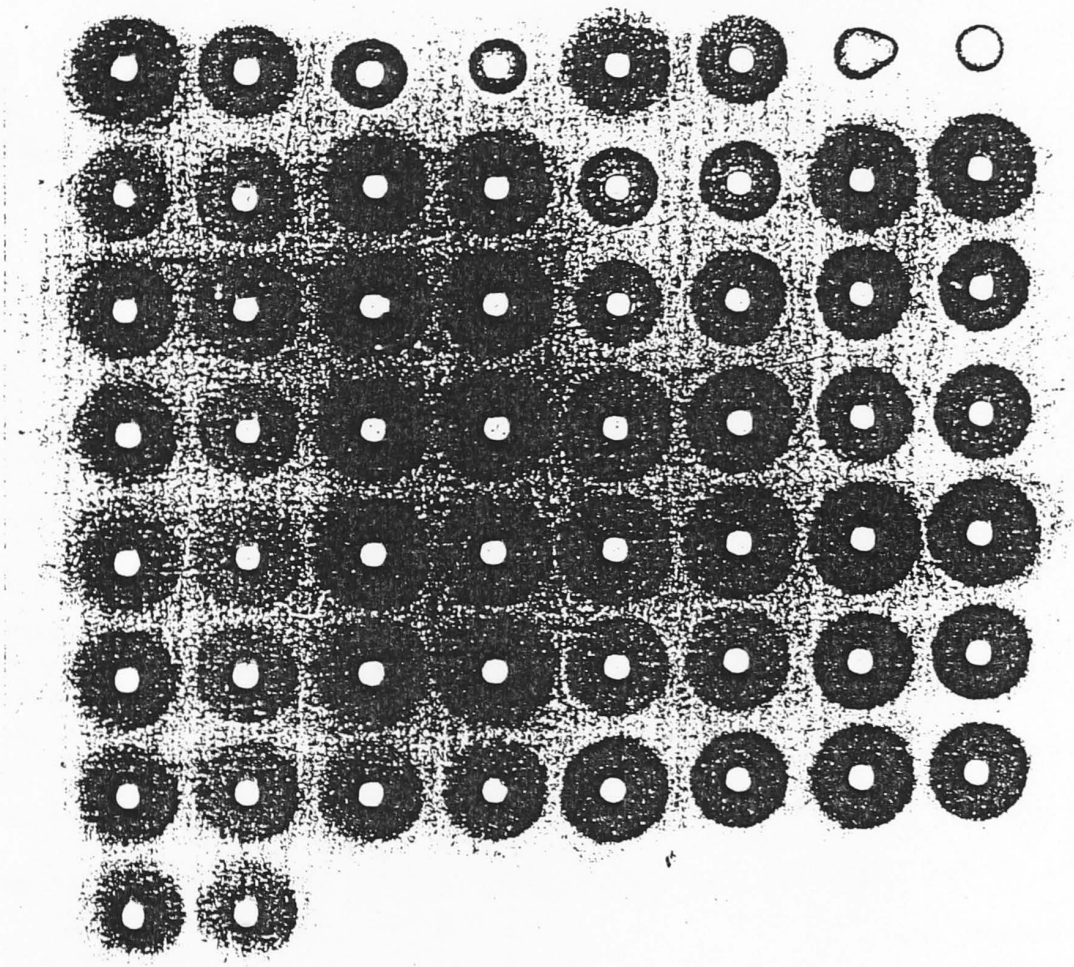


Figure 6.3.2.b

Figure 6.3.3 Analysis of the stained ring diameters. Solid lines are least-squares regression lines for control Adh^F line C5 (upper) and Adh^S line C8 (lower).The circles represent concentrations of 100, 50, 25 and 12.5. The stained ring diameter measured in the same plate was converted to log concentration by using the standard least-squares regression lines. In the figure a horizontal broken line is drawn at diameter 12.65, The log concentrations giving that ring diameter were 1.37 for the upper line and 1.45 for lower line (from the dropped broken line).

Figure 6.3.3

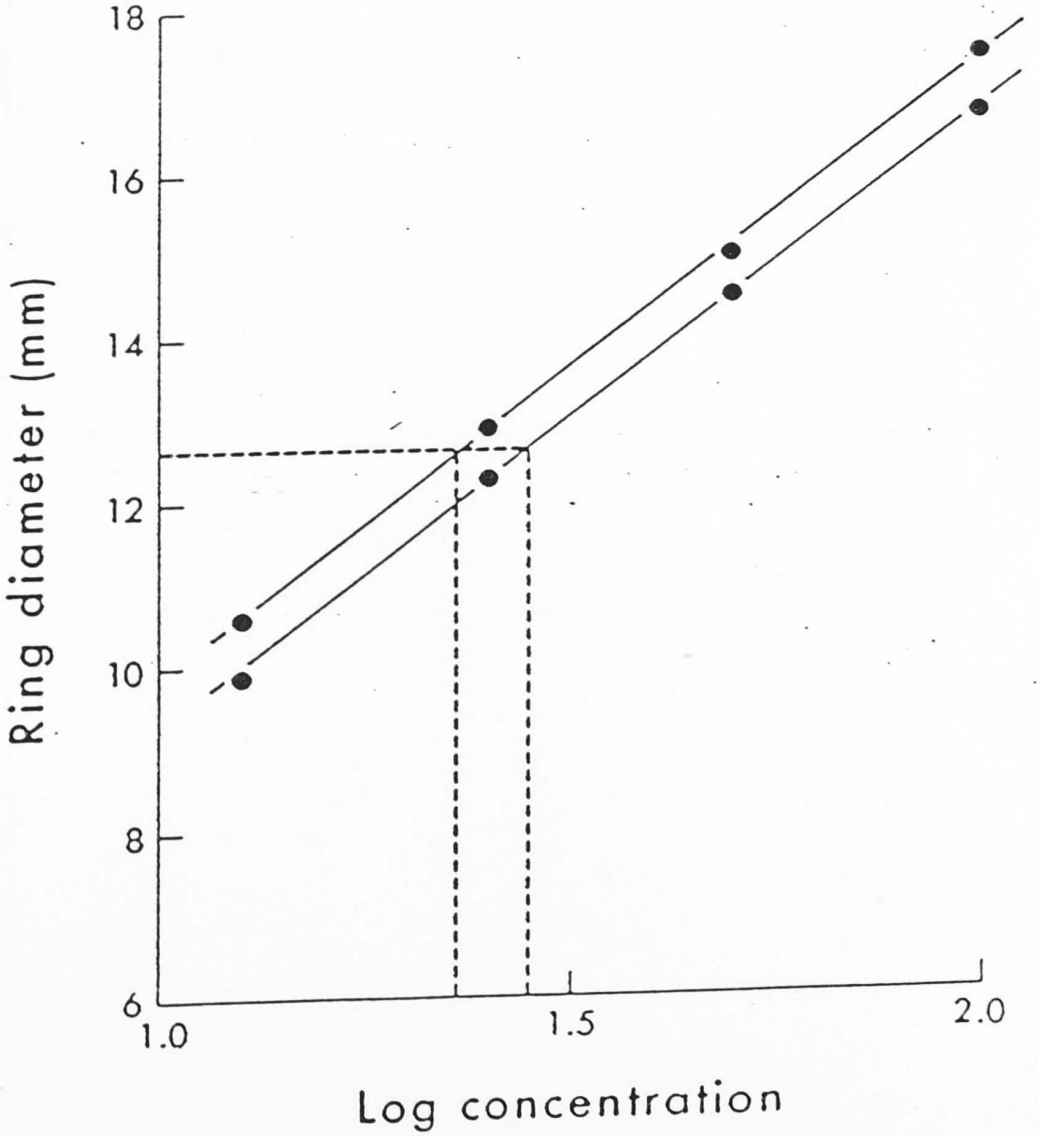


Figure 6.3.4.a The relationship between ADH-F activity and ADH protein amount.

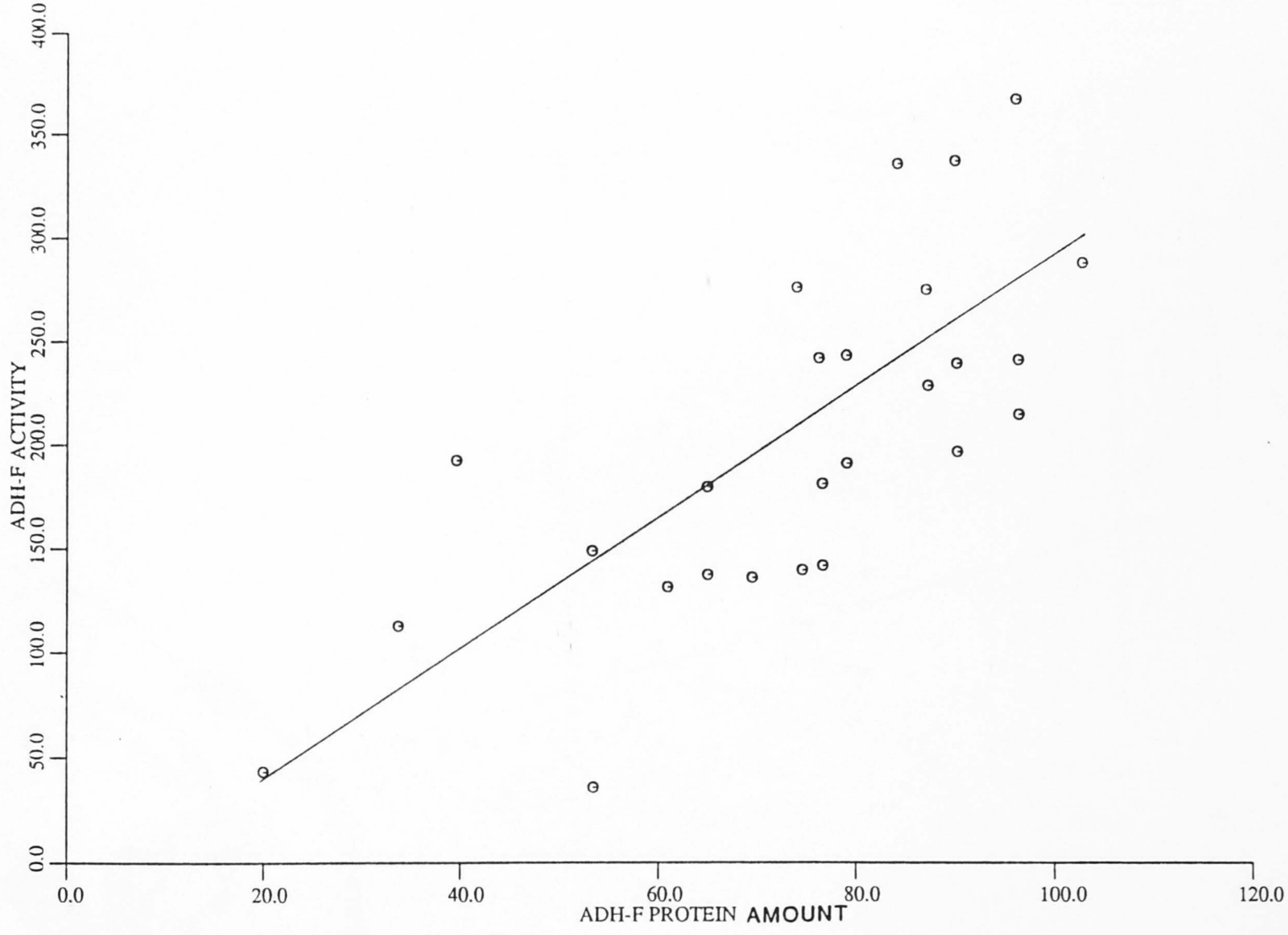

ADH-F ACTIVITY
0.0
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0
0.0
20.0
40.0
60.0
80.0
100.0
120.0
ADH-F PROTEIN AMOUNT

Figure 6.3.4.b The relationship between ADH-S activity and ADH protein amount.

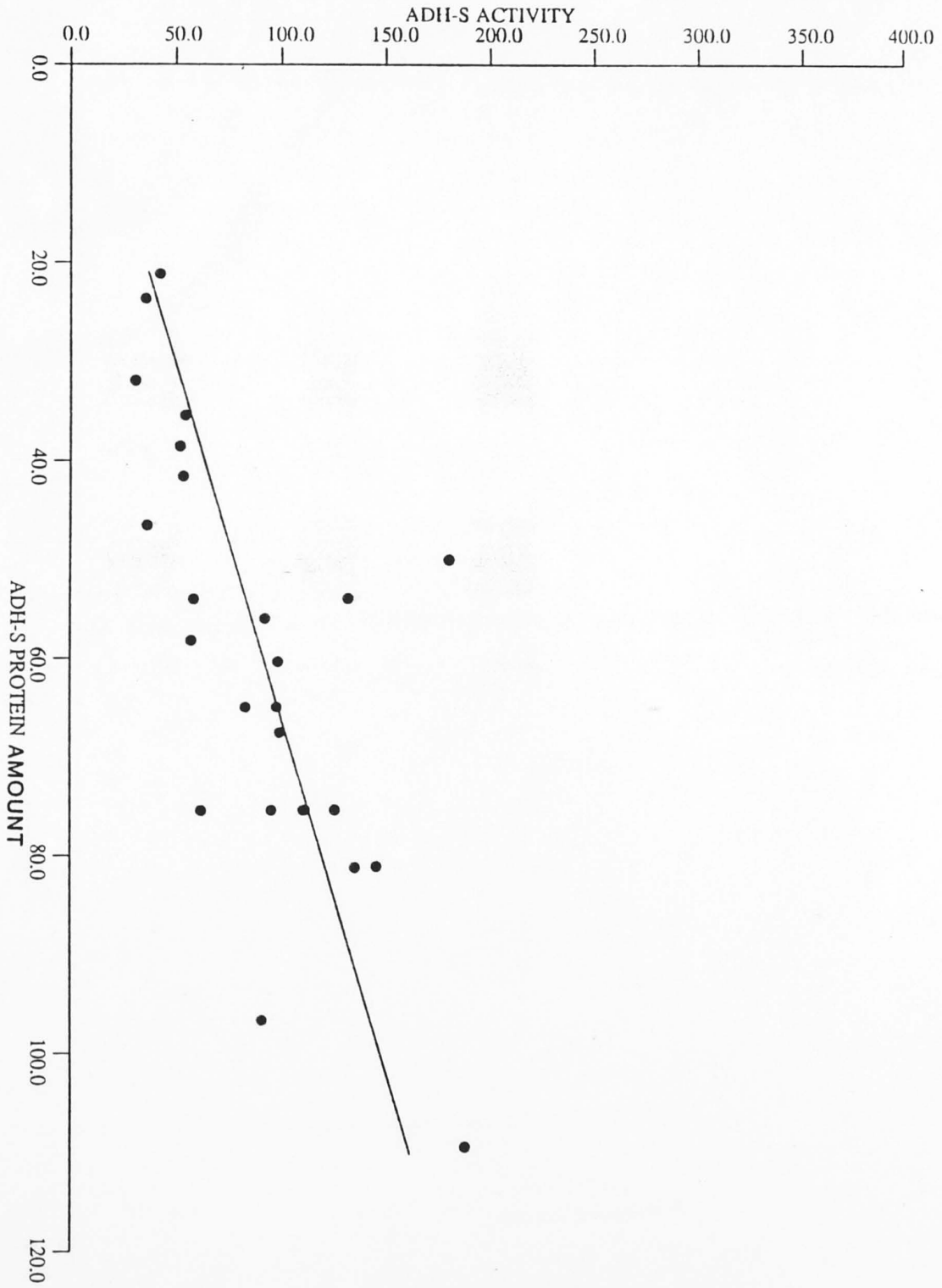

ADH-S ACTIVITY
0.0
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0
ADH-S PROTEIN AMOUNT
0.0
20.0
40.0
60.0
80.0
100.0
120.0

Figure 6.3.4.c The relationship between ADH-FCHD activity and ADH protein amount.

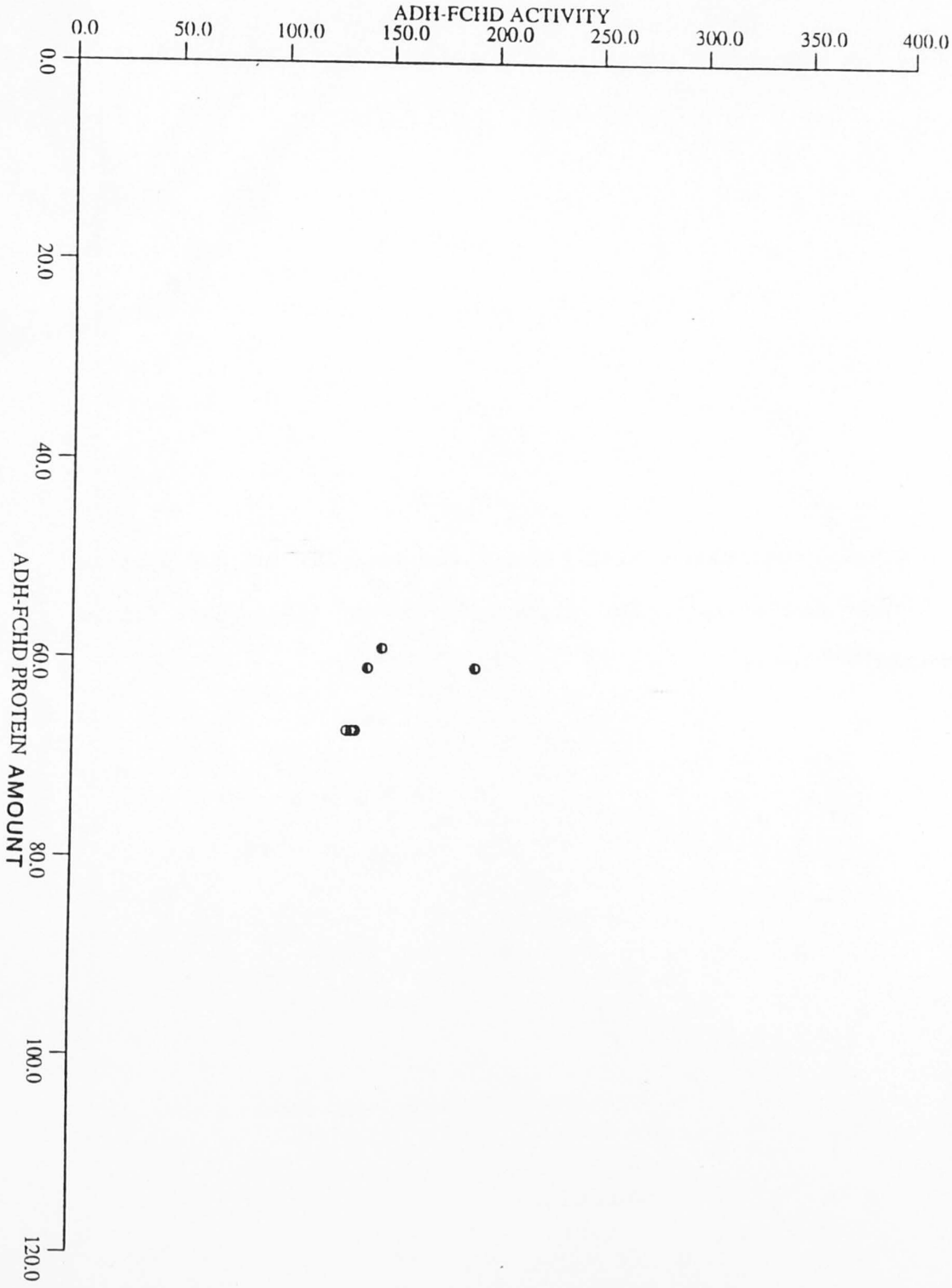
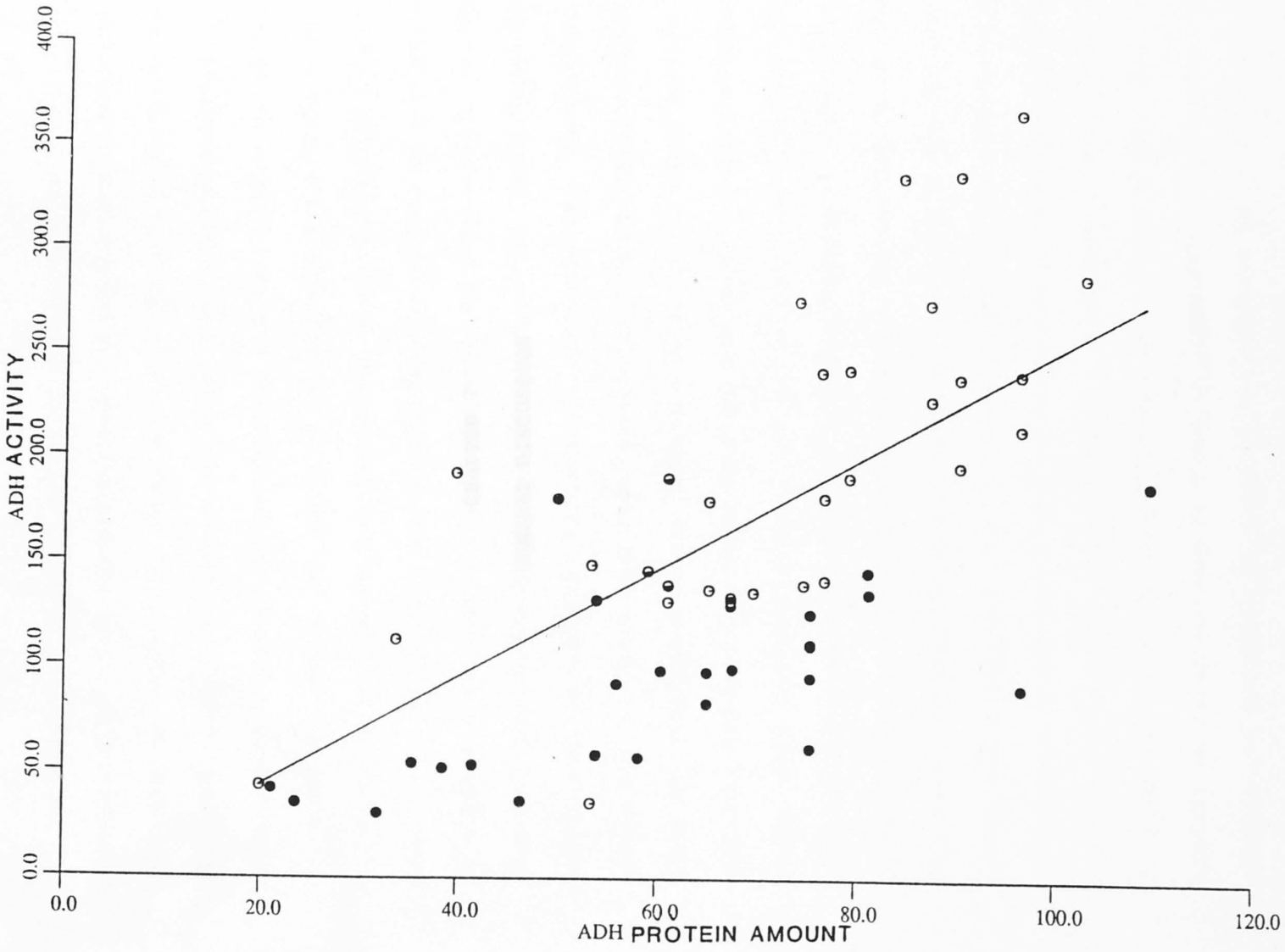

ADH-FCHD ACTIVITY
0.0
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0
ADH-FCHD PROTEIN AMOUNT
0.0
20.0
40.0
60.0
80.0
100.0
120.0

Figure 6.3.4.d The relationship between ADH activity and ADH protein amount. Open circle-*Adh*^{F} lines; Filled circle-*Adh*^{S} lines; Half filled circle-*Adh*^{FChD} lines.


ADH ACTIVITY
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
0.0
20.0
40.0
60.0
80.0
100.0
120.0
ADH PROTEIN AMOUNT

CHAPTER 7

GENERAL DISCUSSION

Chapter 7. General Discussion

The allozyme frequency survey of populations from China, which were chosen for their similar latitudes to populations in Australia in the southern hemisphere, revealed the geographic patterns of eight allozyme loci. The patterns in China show some similarities and differences compared with the patterns previously found in Australia and other continents. The low level of polymorphisms in *Odh, Acph, Pgd and Pgm* (the average frequencies of the common alleles at these loci were 99.9%, 98%, 98% and 94% respectively) is similar to previous data from Eurasia and Japan, whilst the level of polymorphism for these loci is higher in North America and Australia (89.1% and 95.4%, 94.6% and 98.3%, 72% and 77%, 85% and 85% respectively). Populations in Europe and Asia have been under selection of temperate climate for at least 10-15 thousand years, however populations in North America and Australia had been introduced from the mixture of African and European flies only centuries ago. The different levels of allozyme polymorphism probably reflect the different history of colonization of these continents by *Drosophila melanogaster*. For the loci which show a high level polymorphism similar patterns were found in the Chinese and Australian populations. The frequency of *Gpdh* was not correlated with latitude in either the Chinese or Australian populations, and a *Gpdh* cline was only found in North America (Oakeshott *et al.*, 1982). The frequencies of Adh^S and $G6pd^F$ showed latitudinal clines which were similar in direction and in shape to those found in Eurasia, North America and Australia in spite of the differences in the history of colonization and agriculture between these continents. These clines cannot be reasonably explained by any neutral random drift model, and

they are more likely maintained by some kind of selective forces which vary latitudinally.

An unexpected observation is the direction of the *Est-6*$^{1.00}$ cline found in China which is opposite to that found in Eurasia, North America and Australasia. In these continents the frequency of *Est-6*$^{1.00}$ increases with increasing latitude. Data from the study of selection on the *Est-6* locus were often contradictory. Seasonal changes in *Est-6*$^{1.00}$ frequency indicated that this allele is favoured in cooler climates (Franklin, 1981). However the data from laboratory experiments of the effects of temperature on the frequency of *Est-6*$^{1.10}$ (Birley and Beardmore, 1977) showed the frequency is negatively correlated with temperature, and studies of thermostabilities indicated that the enzyme produced by *Est-6*$^{1.00}$ is more stable than that produced by *Est-6*$^{1.10}$ between 30° - 50° (Danford and Beardmore, 1979).

In a recent study White *et al.* (1988) found that using β-napthyl acetate as substrate EST-6$^{1.00}$ has a higher Vmax at all temperatures tested (10, 15, 20, 25 and 30°C). The Km values are similar for the allozymes at 10-20°C, but between 20 and 25°C the Km of Est-6$^{1.00}$ increased significantly whilst the Km of EST-6$^{1.10}$ did not change. Therefore the Km of EST-6$^{1.00}$ is significantly more efficient than EST-6$^{1.10}$ between 10° and 20°C, and there is no significant difference at 25 and 30°C, although the Km of Est-6$^{1.00}$ is still higher than EST-6$^{1.10}$. The peak catalytic efficiency for EST-6$^{1.00}$ occurs at 15°C, while that for EST-6$^{1.10}$ occurs between 20 and 30°C. The catalytic efficiencies of the two allozymes vary with temperature in a way that is consistent with the clinal variation observed in natural populations from North America and Australia (Oakeshott *et al.*, 1981) but not China.

A possible explanation for the reversed *Est-6* cline in China may be linkage disequilibrium between $Est\text{-}6^{1.00}$ and the inversion *In(3L)P*. The inversion *In(3L)P* shows a cline in the opposite direction to $Est\text{-}6^{1.00}$ in North America (Mettler, Voelker and Mukai 1977), Australasia (Knibb *et al.*, 1981) and Japan (Inoue and Watanabe, 1979). The frequency of this inversion in North American populations varied from 6.7% (above latitude 40°N) to 18.2% (between 25 - 30°N), in Australian populations from 4.6% (between 35 - 40°S) to 28.6% (above 25°S), and in Japanese populations the frequency of *In(3L)P* was quite low except in a population from southern Japan (Okinawa 27.5°N) where the frequency reached 40%. The distribution of inversion *In(3L)P* in Chinese populations is unknown. In southern China the frequency of this inversion might reach quite high levels since southern China is closer to the equator than southern Japan. This could account for the reversed *Est-6* cline in China. The investigation of chromosome inversions in Chinese populations of *Drosophila melanogaster* will be a valuable project for the future.

The *Adh* cline is the most consistent allozyme cline, with Adh^F frequency increasing with increasing latitude, and was found in all continents studied (Oakeshott *et al.*, 1982; David *et al.*, 1986, 1988) including North America, Australasia, Europe, Africa and Asia. A search for possible linked factors which may be responsible for the *Adh* latitudinal cline involved investigation of geographic patterns of ADH activity and restriction endonuclease variation in the *Adh* gene region in my study. ADH activity assays for Australian and Chinese populations of *Drosophila melanogaster* revealed that on average ADH-F has a two- to threefold higher activity level than ADH-S in populations from both hemispheres. This is consistent with previous reports from other geographic regions (Laurie-Ahlberg *et al.*, 1980;

Birley and Marson, 1981; Anderson and Gibson, 1985; Aquadro *et al.*, 1986). The difference in ADH activity between the two alleloenzymes is mainly due to the amino acid substitution in the structural gene, although there are some trans-acting factors (Hewitt *et al.*, 1974; Ward 1975; McDonald and Ayala, 1978; King and McDonald, 1987) and *cis*-acting regulatory elements (McKay, 1981; Maroni and Laurie-Ahlberg, 1983) affecting ADH activity.

Laurie-Ahlberg and Stam (1987) used P-element transformation to generate recombinants *in vitro* in the *Adh* region, and then tested the effects of particular regions on expression *in vivo*. The region was mapped to a 2.3kb *Hpa*I/*Cla*I fragment which includes the whole *Adh* coding sequence, and 3′ flanking sequence but excludes the 5′ flanking sequence of the distal transcriptional unit. The differences in this region between typical Adh^F and Adh^S were only three nucleotide substitutions (Kreitman, 1983): one substitution is the ADH-F/ADH-S amino acid replacement (at 1490), the others are two silent substitutions (at 1443 and 1527). This leads to a deduction: if the selection on ADH activity is the cause of the *Adh* cline, then the selection must act on the structural gene instead of on linked modifiers. Data from the present study indicate that the variation in ADH activity within the genotypes Adh^F or Adh^S (which is due to polymorphic modifiers) was not correlated with latitude. This suggests that the *Adh* cline is not related to ADH activity modifier loci.

A common feature of the worldwide geographic differentiation of *Adh* gene frequency is the high frequency of Adh^F in cool temperate populations: 95% in France, 86% in North-East China, 80% in North Japan and about 80% in southern Australia (except the island of Tasmania). Populations of *Drosophila melanogaster* in these areas do not breed over the winter months. Each year the cool temperate

populations experience a population bottleneck or founder effect. The Adh^F gene, or some linked factor, may confer an advantage for survival in cold temperatures since Adh^F frequency remains high in cool temperate regions. There are some data supporting this possibility: Pipkin *et al* (1973) analysed the relationship between *Adh* frequencies and climatic variables in 14 natural populations along the gulf of Mexico coast, and the central highlands of Mexico and Texas. They found regressions of *Adh* frequency on extreme minimum monthly temperature (Tmin) for a ten year period was significant at 1% level and on mean temperature at the 5% level, but not significant on extreme maximum monthly temperature. A similar relationship was found in 24 Spanish populations by Malpica and Vassallo (1980). Johnson and Powell (1974) reported a higher Adh^F frequency for the survivors of cold shock (0°C for 24-48hr) in the laboratory, but the significant regression of *Adh* frequency on Tmin was not confirmed by worldwide surveys. In contrast McKenzie and McKechnie (1981) reported a positive correlation between Adh^F frequency and temperature (varied from 8° -22°C) in the cellar population at the "Chateau Tahbilk" vineyard; this result showed that Adh^F flies were at a disadvantage in cool temperatures. They also found that heterozygote males have a greater mating propensity and heterozygote females have a significantly higher fertility than either homozygote. In addition, heterozygotes have higher survival rate at 35°C (Van Delden and Kamping, 1980) and the alcohol tolerance and relative fitness of Adh^F/Adh^S heterozygotes on ethanol containing media are equal to Adh^F homozygotes (Oakeshott *et al.*, 1980; Gibson and Oakeshott, 1982). These data suggest a higher heterozygote fitness which prevents frequency changes in populations fixing one allele, and maintains nearly all populations worldwide polymorphic for the two alleloenzymes. The main finding from my data

is that the Adh^S cline occurs on every continent so far studied and this evidence provides strong support for selection even though the mechanism remains unknown.

Another finding from my work on allozyme distributions which deserves comment is that a number of rare alleles were found in my survey which had previously been described in populations on other continents. This raises questions about their origins and maintenance.

A rare *Gpdh* allele, $Gpdh^{UF}$, was found in a Chinese population from a winery in Xiamen, at an unusually high frequency (13%). Alleles with the same electrophoretic mobility previously were found in a North American population at a estimated frequency 0.01% (Lee *et al.*, 1980), in an African population from Cameroon at a frequency 0.7% (David, 1982) and also in an Australian population at a very low frequency (John Gibson, personal communication). The product of $Gpdh^{UF}$ has the same molecular weight (34,000) as $Gpdh^F$ and $Gpdh^S$ but exhibits a different isoelectric point in two-dimensional electrophoresis. At least one charged amino acid substitution is believed to have occurred in this rare allele (Lee *et al.*, 1980) although amino acid composition analysis did not identify the exact amino acid substitution (Niesel *et al* 1982; Bewley, 1983). This rare allele was not found in the two populations north and south of the Xiamen population. This locally high frequency of $Gpdh^{UF}$ may be a result of a mutation which has been copied many times in the small inbreeding winery population and the frequency of this allele reached a quite high level. It is unlikely that this mutation occurred repeatedly in the Xiamen population since $Gpdh^{UF}$ occurred in populations from other continents at very low frequency. Little is known about the molecular structural change, or about the fitness of homozygotes and heterozygotes for this rare allele.

Two rare *Adh* alleles, the Adh^F heat resistant form and *Adh* null alleles, were found in my study.

The discovery of high frequencies of Adh^F heat resistant alleles in populations from southern China is an interesting result from my study. These heat resistant alleles show similarities in thermostability, ADH activity, activity ratio and amount of ADH protein in adult flies. They also show the lowest level of nucleotide substitution and haplotype diversity compared with Adh^F and Adh^S lines, and they share characteristics with the Adh^F heat resistant alleles previously found in North America, Europe and Australia. These data strongly suggest that this allele had a recent origin. It is possible that this allele arose in south China and was then spread worldwide via the agency of man.

Another possibility is that the heat resistant alleles were derived from a mutation (amino acid substitution of serine for proline at residue 214 in Adh^F) which occurred independently, but repeatedly in different geographic regions. That means residue position 214 is a "hot sport" for mutation, so that the frequencies of heat resistant alleles should be similar in most populations.

Some data from the estimated time of allele divergence may provide more information about the history of this allele. Aquadro *et al.*(1986), using the data from linkage disequilibrium between the variant *Bam*HI(-7.2) and Adh^S in American populations, estimated the divergence time of Adh^S from Adh^F. They estimated that the rate of recombination over the 7.3kb of the *Adh* gene region was approximately $3.6X10^{-5}$ events/kilobase/generation, the current level of linkage disequilibrium in U.S. would take approximately 7880 generations under selective neutrality, or about 2000 years. The divergence time of Adh^{FChD} from Adh^F should be more recent, since it is derived from

Adh^F, so that it is quite possible that the heat resistant allele originated in southern China recently and its spread is still occurring. Using sequencing data a very different divergence time was estimated. Ashburner *et al.* (1984) estimated that Adh^F arose from Adh^S 1-3 million-yr ago, and Collet (1988) estimated that the time for Adh^{FChD}/Adh^F divergence is 0.26-0.473 million-yr ago. The latter estimation was based on the sequence of a single Adh^{FChD} allele. It is believed that the colonization of Eurasia by *Drosophila melanogaster* occurred only 10-15 thousand years ago or bit earlier (David and Capy, 1988; Lachaise *et al.*, 1988), so this would suggest that Adh^{FChD} did not first arise in China.

Logically, it might be expected that this allele has a selective advantage in warmer environments according to its thermostability *in vitro*. *In vivo* biochemical and physiological fitnesses of a heat resistant allele were studied by Heinstra *et al.* (1987). They studied the differences in metabolism and viability between different *Adh* genotypes in third-instar larvae, and found that after larvae were exposed to acetone or ethanol (5% v/v), propan-2-nol or ethanol respectively were found in the body of larvae. ADH-71k (produced by a heat resistant Adh^F allele found in a laboratory population in the Netherlands, Thorig *et al.*, 1975) has the highest rate in eliminating propan-2-nol and ethanol *in vivo*. The larvae with the Adh^{71k} genotype have a significantly lower accumulation of propan-2-nol and ethanol compared with larvae of other genotypes. Rates of accumulation of ethanol *in vivo* are negatively associated with larval-to-adult survival. When mid third-instar larvae were transferred to medium containing 10% ethanol, the average percent of eclosed individuals were 69.2% of Adh^{71k} genotype, 56.2% of Adh^F, 42% of Adh^S and zero of null *Adh*.

The difference in substrate specificity between ADH-71k and other alleloenzymes was reported by Eisses *et al.* (1985b) and Eisses *et al.*, (1986): ADH-71k oxidized sarcosine *in vitro* as well as *in vivo*, which showed differences in substrate competition between genotypes. Whether the above metabolic and physiological properties are shared by the other Adh^F heat resistant lines found in China, Australia and Europe needs further investigation. Data from this kind of investigation will be useful to fully understand the fitness and history of heat resistant alleles.

The *Adh* null alleles found in Coffs Harbour, and Tahbilk (near Melbourne) populations have a different molecular structure from that found in Tasmanian populations (J.B. Gibson personal communication). Ch14 and W449 (from Coffs Harbour populations) are similar in structure, both have a 440bp deletion in exon 2. A null allele found in the Tahbilk population has a 320bp insertion in intron I (Alex Agrotis, personal communication of unpublished data). The Tasmanian null alleles share similar characteristics of biochemical, immunological and molecular properties (Freeth, 1986; Freeth and Gibson 1985) and share the same chromosomal haplotype (see chapter 4). They are highly likely derived from a single mutation which spread throughout the Tasmanian populations. The probability that this mutation occurred repeatedly in different populations is very low. The relatively high frequency of null alleles in Tasmanian populations might result from recent winter bottlenecks. There is no evidence for the existence of the Tasmanian null allele on the Australian mainland. This suggested that there is very limited migration of *Drosophila melanogaster* between the island of Tasmania and the Australian mainland.

The investigation of restriction endonuclease variation in the region of the *Adh* gene in Australian and Chinese populations failed to find any variant which shows a consistent latitudinal cline parallel to the *Adh* cline. The pattern of restriction endonuclease variation found in the present study is consistent with previous work. Insertions/deletions mainly occurred in flanking sequences. Collated data from Langley (1982), Birley (1984), Cross and Birley (1986) and Aquadro *et al.* (1986) showed that 70% of insertions (larger than 50bp) occurred in a 0.23kb *Eco*RI/*Sal*I fragment of the 3' flanking sequence (see Figure 3.4.1). This cluster of insertions may be due to the susceptibility of the 0.23kb fragment to insertion or due to natural selection. The sequence of this fragment is not known, but it may be recognised by some mobile elements. If natural selection is the cause, then the selective constraint on this fragment must be less compared with other regions. Actually the insertions found in this fragment did not show any effect on ADH activity. Large insertions were rare and local, with some sharing sequence homology with those mobile elements which have an abundant copy number in the genome. None of the insertions in the region of the *Adh* gene were homologous to *P* elements. Most of the insertions have little effect on the expression of the *Adh* gene. Apparently insertions/deletions are not responsible for the *Adh* cline.

Some polymorphic restriction endonuclease sites and insertions/deletions are ubiquitous, such as *Xho*I(1.2), *Bam*HI(-7.2), *Hin*III(-3.0), and *Eco*RI(9.0), 0.4kb insertion and 0.2kb deletion. They seem to be ancestral polymorphic variants and have been spread worldwide by migration. Others are more local and reflect divergence between populations, which perhaps occurred more recently.

The linkage disequilibria between variants provide an indication of population structure. The most common linkage disequilibrium between variation in the *Adh* gene region found in present and previous studies is between *Bam*HI(-7.2) and Adh^S. Two explanations could be applied to this linkage disequilibrium (Cross and Birley, 1986). In the first explanation, the Adh^F allele arose on a Adh^S bearing chromosome without the *Bam*HI(-7.2) site. The frequency of this ancestral Adh^F allele increased because of its selective advantage in temperate regions so that this linkage disequilibrium is a product of the "hitchhike" effect. The second explanation is that this linkage disequilibrium is maintained by selection, it may be favoured by selection. Data from my study supports the first explanation. There is no consistent relationship between the *Bam*HI(-7.2) site and ADH activity in Australian and Chinese populations (see chapter 6), and the linkage disequilibrium was looser in Chinese populations and non-significant in some Australian populations. Laurie-Ahlberg and Stam (1987) used P-element-mediated transformation to identify variants affecting the expression of *Adh* gene and their results rule out involvement of any variants in the 5′ flanking region. This linkage disequilibrium may be expected to become weaker and weaker in the future.

The comparison of restriction map variation between Australian and Chinese populations of *Drosophila melanogaster* provided more information about the different histories of colonization by *Drosophila melanogaster* of the two countries. On average higher levels of nucleotide substitution and haplotype diversity were found in the "new" Australian populations than in the "old" Chinese populations. No environmental variables were found to be correlated with these

differences, although the levels vary between populations from different geographic regions.

The levels of nucleotide substitution and haplotype diversity are higher in Adh^S lines than in Adh^F lines and lowest in Adh^{FChD} lines in the populations from both countries. This is consistent with the allelic phylogeny derived from sequence data (Kreitman, 1983; Ashburner *et al.*, 1984; Bodmer and Ashburner, 1984; Collet, 1988).

Taken together the data obtained in the present study, suggest some conclusions might be drawn. The geographic allozyme data from mainland China collated with data from Japanese populations filled the Far East "gap" in the data set for the worldwide patterns, and the generality of *Adh* and *G6pd* clines was confirmed in the Chinese populations. The consistent *Adh* and *G6pd* clines in different continents strongly suggested that these clines are maintained by natural selection. Considering the different history of colonization by *Drosophila melanogaster* and the variety of agricultural environments on the different continents, neutral genetic drift is unlikely to be the cause. The reversed *Est-6* cline in populations from mainland China shows that this cline cannot be considered to be consistent and the cause needs further investigation of the linked inversion *In(3L)P* pattern in Asia. The investigation of ADH activity and restriction endonuclease variation in the region of *Adh* gene in populations from Australia and China indicated that any selection maintaining the *Adh* cline does not act on ADH activity modifier loci or on closely linked restriction endonuclease variation. The different levels of allozyme polymorphism, ADH activity, nucleotide substitution, and haplotype diversity in the populations from Australia and China could be mainly due to the different history of *Drosophila melanogaster* colonization in Australia and China.

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